ANNUAL REVIEW OF MICROBIOLOGY

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VOLUME 6

1952

ANNUAL REVIEWS, INC.
STANFORD, CALIFORNIA, U.S.A.

ANNUAL REVIEWS, INC.
STANFORD, CALIFORNIA, U.S.A.

FOREIGN AGENCIES

H. K. Lewis & Company, Limited
136 Gower Street
London, W.C. 1

Maruzen Company, Limited
6, Tori-Nichome Nihonbashi
Tokyo

PRINTED AND BOUND IN THE UNITED STATES OF AMERICA BY
GEORGE BANTA PUBLISHING COMPANY

PREFACE

The preface to this *Review* enables us to once again express our thanks to those who have collaborated in the authorship of this volume. Their surveys of advances in the various fields of microbiology are important contributions to a better understanding of microorganisms and their behavior in animate and inanimate environments. The Editorial Committee continues to welcome criticism and also suggestions as to authors and topics for future volumes.

The Editorial Committee is composed of five members with one member retiring each year. We wish to extend our thanks to Dr. C. B. van Niel, the retiring member for the current year, for the time and effort he has so graciously contributed to the development of the *Review*.

It is a pleasure, as in past years, to extend our appreciation to the office staff of Annual Reviews and to the George Banta Publishing Company for their painstaking care and co-operation in the preparation of this *Review*.

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METABOLISM OF MICROORGANISMS

By Herman C. Lichstein

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This review covers in part the literature appearing during 1951, and is restricted primarily although not entirely to bacterial metabolism. As in previous years, the field of metabolism moved too rapidly for total coverage to be achieved in the space limitations imposed. Certain areas of metabolism are treated more extensively than others because of their importance, and attempts are made when possible to relate papers and to comment on their significance.

Fermentation

Heterolactic fermentation.—Gibbs & DeMoss (1) and DeMoss, Bard & Gunsalus (2) have suggested a departure from the Meyerhof-Embden scheme in the formation of ethanol by *Leuconostoc mesenteroides*. Resting cell suspensions yielded fermentation balances which indicated that glucose was fermented to one mole each of lactate, ethanol, and CO_2. In contrast, dried cell preparations produced one mole of lactate per mole of glucose, thus accounting for but half of the carbon of the substrate. Diphosphopyridine nucleotide (DPN) linked dehydrogenases for ethanol, lactate, 2,3-butyleneglycol, and 3-phosphoglyceraldehyde were demonstrated in cell-free extracts. Aldolase and isomerase did not appear to be present in this organism, thus suggesting a departure from the Meyerhof-Embdem scheme and the presence of a new glycolytic mechanism. Isotope experiments confirmed this conclusion. In a subsequent communication, ethanol production by *Pseudomonas lindneri* was studied (3). The data based on both isotope and enzyme experiments are consistent with an interpretation that each of the two moles of ethanol was derived from glucose by a different pathway. They suggested that an anaerobic *Zwischenferment* shunt mechanism yields CO_2 and a pentose phosphate from glucose-6-phosphate. The pentose is degraded by a C_2-C_3 cleavage where the C_2 fragment derived from carbons two and three of glucose is the precursor of one mole of ethanol, while the C_3 moiety is converted to pyruvate. The pyruvate is then decarboxylated by carboxylase to CO_2 (carbon four of glucose) and acetaldehyde which is reduced to ethanol (carbons five and six of glucose).

Acetoin production.—Three groups of investigators have reported on acetoin formation from pyruvate by lactic organisms. Rowatt (4) found that *Lactobacillus plantarum* converted pyruvate quantitatively to levorotatory acetoin and CO_2 at pH 4. The factors affecting the rate of this reaction most markedly were pH, glucose in catalytic quantities, thiamine, and phosphate. Similar findings were reported by Nossal (5) working with *L. arabino-*

sus. A more complete study was made by Dolin & Gunsalus (6) with *Streptococcus faecalis*. Of interest is the fact that the acetoin forming system in this organism is practically undetectable in resting cell suspensions, which oxidize pyruvate to acetate and CO_2. Cell-free extracts, however, catalyze the breakdown of pyruvate predominately to acetoin and CO_2. The acetoin system, by ammonium sulphate precipitation and dialysis against citrate buffer, was rendered almost completely inactive in the absence of cocarboxylase and manganese. The extracts contained an active α-acetolactate decarboxylase, and acetaldehyde did not apparently serve as an intermediate. Indirect evidence was obtained for the function of divalent ions in the α-acetolatate decarboxylase system. Although no attempt was made to isolate an acetolactate forming system in this organism, the assumption was made that the mechanism of acetoin formation proceeds via α-acetolactate.

At present there appear to be three mechanisms for acetoin formation: (*a*) the system in yeast for which one mole of pyruvate and one of acetaldehyde are required; (*b*) the system in various animal tissues for which one mole each of pyruvate and acetaldehyde, or two moles of acetaldehyde, act as substrates; and (*c*) the system found in some bacterial species for which two moles of pyruvate act as substrate without the intermediate occurrence of free acetaldehyde, to form one mole of acetoin and two moles of CO_2. Juni (7) resolved a system in *Aerobacter* into two components, one which forms α-acetolactate and CO_2 from pyruvate, and a second which decarboxylates the acetolatate to acetoin and CO_2. Clarification of this mechanism will proceed more rapidly when the acetolactate decarboxylase is studied in more detail in the absence of the pyruvate decarboxylase system.

Propionic acid fermentation.—Kohlmiller & Gest (8) observed that the nonsulfur, purple, photosynthetic bacterium, *Rhodospirillum rubrum*, can ferment externally supplied substrates anaerobically in the dark. The fermentation of pyruvate appeared to be typical of the propionic acid fermentation. The differences between the light and dark fermentations are discussed.

Decarboxylation of succinate appears to be the mechanism of propionate formation in *Veillonella gazogenes* (9) as well as in the genus *Propionibacterium*. Barban & Ajl (10) presented data obtained with *P. pentosaceum* which suggest that the succinate decarboxylase system is reversible. When $C^{14}O_2$ was incubated at pH 5.8 in the presence of inactive succinate, fumarate, and malate, there was incorporation of radioactivity into the carboxyl groups of succinate. Fumarate was completely inactive, some activity was recovered in malate, while active propionate was the major volatile end-product.

Formate decomposition.—Stadtman & Barker (11) studied a new formate-decomposing organism, *Methanococcus vanneilii*. This motile coccus converts formic acid to methane, CO_2, and H_2, but appears incapable of obtaining energy from any other organic compound. The reversibility of the phosphoroclastic reaction and some properties of this system are discussed by Strecker (12).

The often observed phenomenon that the presence of nitrate in the growth medium prevents the formation of H_2 by *Escherichia coli* was elucidated by Billen (13) with the finding that nitrate inhibited the formation of formic dehydrogenase, formic hydrogenlyase, and hydrogenase. Increasing concentrations of hydrolyzed casein antagonized the inhibitory effect of the nitrate. The possibility that nitrate acts by stimulating nitratase formation, thus eliciting the utilization of amino acids otherwise available for the formation of these enzymes, is discussed. That the formation of these enzyme systems is conditioned by the presence of certain amino acids in the growth medium was demonstrated by Billen & Lichstein (14). Formation of the formic hydrogenlyase system was stimulated by several groups of amino acids and by glutamic acid singly, while formic dehydrogenase activity was increased when glutamate, tyrosine, or methionine was supplied. Little or no activity resulted when an inorganic nitrogen source was supplied during growth. These results were confirmed by use of nonproliferating cells. It would appear that although *E. coli* is capable of synthesizing amino acids from inorganic nitrogen compounds certain enzymes are not produced unless definite amino acids are present preformed in the medium. The data on nitrate inhibition as well as the specificities in amino acid requirements lend weight to the identity of the formic hydrogenlyase enzyme.

Methane fermentation.—Stadtman & Barker (15, 16) have continued their excellent contributions to our knowledge of the mechanism of the methane fermentation. Two new species of methane producing microorganisms that carry out incomplete oxidation of fatty acids were described, and employed in tracer studies with C^{14} labeled compounds in an attempt to ascertain the mechanism by which various lower fatty acids are degraded and methane produced. *Methanobacterium suboxydans* was found to convert valeric acid to propionic and acetic acids by means of beta oxidation and in a similar fashion butyric and caproic acids were oxidized to acetate:

$$2CH_3(CH_2)_3COOH+CO_2+2H_2O\rightarrow 2CH_3CH_2COOH+2CH_3COOH+CH_4 \quad 1.$$

$$2CH_3(CH_2)_2COOH+2H_2O+CO_2\rightarrow 4CH_3COOH+CH_4 \quad 2.$$

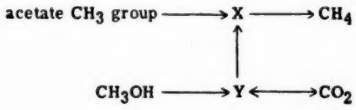
M. propionicum oxidizes propionate to CO_2 and acetate

$$4CH_3CH_2COOH+2H_2O\rightarrow 4CH_3COOH+CO_2+3CH_4 \quad 3.$$

in such a manner that the carboxyl carbon gives rise to CO_2 and the alpha and beta carbons yield acetate. Since *M. suboxydans* is unable to oxidize either acetate or propionate and no exchange of carbon occurs between CO_2 or methane and acetate or propionate, it appears that acetate is not an intermediate in the reduction of CO_2 to methane, although the general CO_2 reduction theory of methane production is still tenable.

In tracer experiments with a strain of *Methanosarcina* which can utilize either methanol or acetate as a sole source of carbon, it was found that the methane was derived from the substrate and that CO_2 reduction was a minor reaction. With methyl labeled acetate, for example, the methane was derived

from the methyl group and the CO_2 came from the carboxyl group. On the assumption that there is only one general mechanism of methane formation and on the basis of facts now available, a scheme which explains the origin of methane from both methanol and CO_2 was presented as follows:



4.

Thus, methanol is transformed probably by oxidation to a hypothetical intermediate common to the paths of methane formation from both methanol and CO_2. Although the identity of X or Y is not known, compound X is distinguished from Y by the fact that it is readily converted to methane but not to CO_2, whereas Y is converted to both. The conversion of Y to X must, therefore be almost irreversible. The scheme does not exclude the conversion of CO_2 to methane during the fermentation of acetate and methanol, and CO_2 reduction may be inhibited by an accumulation of X and Y which are presumably formed more readily from acetate and methanol than from CO_2.

Galactose and fructose fermentation.—Garner & Grannis (17) have contributed to an understanding of galactose fermentation by the isolation of a phosphogalactoisomerase from *Saccharomyces marxianis*. This enzyme, not yet demonstrated in animal tissue, catalyzes the transformation of galactose-1-phosphate to glucose-1-phosphate and requires uridinediphosphoglucose (UDPG) as a coenzyme (18). It appears therefore that the fermentation of galactose proceeds initially as follows:

$$\text{galactose} + \text{ATP} \xrightarrow{\text{galactokinase}} \text{galactose-1-phosphate} + \text{ADP} \qquad 5.$$

$$\text{galactose-1-phosphate} \xrightarrow{\text{phosphogalactoisomerase}} \text{glucose-1-phosphate} \qquad 6.$$

$$\text{glucose-1-phosphate} \xrightarrow{\text{phosphoglucomutase}} \text{glucose-6-phosphate} \qquad 7.$$

Fructose utilization by *Pseudomonas putrefaciens* was reported by Klein (19) although the pathway was not elucidated. Hexokinase activity was not demonstrable nor was there any evidence for the formation of fructose-1-phosphate rather than fructose-6-phosphate.

Pentose fermentation.—Information, hitherto lacking, on pentose metabolism is beginning to become available, although most of the reports have been brief. The hypothesis of an initial cleavage of pentoses into C_3 and C_2 fragments is supported by the work of Lampen, Gest & Sowden (20) with *Lactobacillus pentosus* and 1-C^{14}-D-xylose. This organism is known to produce one mole each of acetic and lactic acids per mole of pentose fermented, and this stoichiometry has been the chief basis for the hypothesis of the C_3-C_2 cleavage. The tracer studies reported in this paper showed that lactic acid contained a negligible amount of C^{14} whereas acetate accounted for

95 per cent of the activity. Since degradation of acetate revealed that all the C^{14} resided in the CH_3 group, it was apparent that the CH_3 group of acetate was derived from the aldehyde carbon of xylose. It was suggested that the cleavage occurs between carbons two and three and that a 2-ketopentose is an intermediate in the fermentation. In a similar study by Rappoport, Barker & Hassid (21), the fermentation of L-arabinose-1-C^{14} by *L. pentoaceticus* in growing cultures was found to yield lactate and acetate with the latter being active. All of the C^{14} was found to be in the methyl carbon of acetate with the same specific activity as carbon one in L-arabinose, thus demonstrating that the methyl carbon of acetate is derived exclusively from carbon one of the pentose. The carboxyl carbon of acetate is derived presumably from carbon two, and lactic acid from carbons three, four, and five. Thus, a direct cleavage between carbons two and three is inferred as with xylose (20), and the immediate products should be glycolaldehyde and glyceraldehyde or their phosphorylated derivatives. Indirect evidence is thus provided for a new type of fission reaction differing in several respects from the classical aldolase reaction between dihydroxyacetonephosphate and acetaldehyde. It is pertinent to note that glycolaldehyde was detected as the 2,4-dinitrophenylosazone in cultures of *Acetobacter* species growing on media containing D-xylose and L-arabinose (22). The specificity of pentose fermentation was studied by Lampen & Peterjohn (23) utilizing suspensions and extracts of *L. pentosus*. Perhaps the most interesting findings were the activation of a ribose-fermenting system by growth in the presence of other pentoses, and the fact that extracts of cells grown on pentoses degraded ribose-5-phosphate whereas extracts of cells grown on glucose did not.

Concerning synthesis, Marmur & Schlenk (24) found that *E. coli* can metabolize ribose by an aldolase mechanism in which glycoaldehyde and glycoaldehyde phosphate are reaction components. The synthesis of pentose by this route was accomplished. Racker (25) demonstrated that cell-free enzyme preparations obtained from *E. coli*, *S. faecalis*, and *Corynebacterium diphtheriae* catalyze the reaction:

$$\text{glyceraldehyde } PO_4 + \text{acetaldehyde} \rightarrow \text{desoxypentose } PO_4, \qquad 8.$$

thus showing that triose phosphate is a common intermediate between ribose and desoxyribose metabolism and suggesting a pathway by which nucleic acids can serve as sources of energy. It would appear then that at least two routes of pentose formation exist, one being the oxidative decarboxylation of hexoses, and the other the synthesis by aldolase condensation of phosphotriose with a C_2 fragment.

Divergence of fermentation.—Watt & Werkman (26) in a study of pyruvate dissimilation by *Micrococcus pyogenes* observed that growth at low final pH in a medium containing glucose enhanced the acetyl methyl carbinol (AMC) type of dissimilation, whereas cells grown on a glucose-free medium brought about principally a dismutation to lactate, acetate, and CO_2. Neutralization of the acid as it was formed resulted in a greatly diminished

yield of AMC suggesting a pH effect rather than a specific action of the carbohydrate.

An interesting case in which the normal alcoholic fermentation by extracts of yeast was diverted to a lactic fermentation upon the addition of a thermolabile constituent of brain tissue extracts was reported by Harpur, Johnson & Quastel (27). No evidence was obtained to suggest that this effect is due to a destruction of a yeast dehydrogenase. They explained their results by the presence in animal tissue extracts of diphosphopyridine nucleotidase and a DPN-linked lactic dehydrogenase. The former enzyme causes a drop in alcoholic fermentation due to destruction of DPN, while the latter enzyme causes both a drop in rate of alcoholic fermentation and a rise in the rate of lactate formation as a result of its competition with carboxylase for the pyruvate produced during the breakdown of glucose. Lactic dehydrogenase of animal tissue is DPN-linked whereas that of yeast is not, which fact is largely responsible for the observation that in a mixture of yeast and animal tissue extracts the pathway of fermentation of glucose is shifted to a considerable extent from alcoholic to glycolysis. Coolidge (28) reported for lactobacilli the conversion of hetero- to homolactic acid fermentation depending on the thiamine requirement of the organism.

Coenzymes.—The structure of uridinediphosphoglucose (UDPG) was elucidated by Leloir (29). It appears to be a glucose-1-phosphate molecule attached to uridine-5-phosphate forming a pyrophosphate link. When treated with an enzyme from *Saccharomyces fragilis*, UDPG yielded about 25 per cent of a galactose-containing compound which may possibly be uridinediphosphogalactose. Clarification of the nature of this compound will contribute to the elucidation of the mechanism of action of the coenzyme. Trucco (30) reported the enzymatic synthesis of UDPG on incubating uridinediphosphate, ATP, and glucose-1-phosphate with Lebedev juice from yeast.

Ohlmeyer (31) obtained results suggesting the presence in fresh yeast of a factor which enables yeast maceration juice at very low levels of orthophosphate to ferment glucose at a rate approximately three times faster than does ATP. This cofactor was purified about 40-fold and exhibited the properties of a nucleotide, perhaps resembling the dinucleotide of Kiessling & Meyerhof (153). It appears to act by stimulating the formation of an unknown phosphate-splitting compound. Further work is required not only to characterize this compound chemically, but also to establish its locus of activity.

That nicotinamide is essential during the process of crushing yeast in order to produce cell-free preparations having high fermentative powers was demonstrated by Hochster & Quastel (32). Nicotinamide apparently prevents the disappearance of cozymase by inhibiting diphosphonucleosidase. The practical use of this observation should aid other workers in preparing highly active juices for metabolic studies.

A detailed study of the speed-controlling reactions in fermentation by quickly dried yeast was made by Meyerhof & Kaplan (33). The introduction

to their paper describes nicely the broad aspects of speed control reactions, while the experimental portion presents data on conditions not previously studied in detail. For example, it was found that when yeast cells were washed and the major portion of the coenzymes and phosphate removed, the addition of 0.5 micromole each of DPN and ATP per ml. was necessary for maximal speed of fermentation. The addition of a dialysate of boiled juice raised the speed of fermentation of washed yeast by 20 to 30 per cent of the maximum obtained in the presence of the usual cofactors. It was suggested that this effect is due possibly to adenine dinucleotides. A phosphate concentration of 0.01 to 0.02 *M* was necessary even when the phosphate was only partly esterified. Fermentation did not occur in the absence of either potassium or ammonium ions or of magnesium, suggesting a deficiency in one or more of the intermediary reactions. Additions of small amounts of phosphoglycerate at optimal potassium phosphate concentrations and pH increased the rate of sugar fermentation and gave rise during the time of phosphoglycerate decomposition to extra CO_2 of about three to five times the amount of the glycerate added. The excess of phosphoglycerate apparently stablizes the various phosphorylating steps of the adenine nucleotides to maintain the most favorable proportions.

Pasteur effect.—An approach to the nature of the Pasteur effect was made by Zetterström, Ernster & Lindberg (34) in studies of the changes in phosphate metabolism induced in *Saccharomyces cerevisiae* by a transition from fermentation to respiration. It was observed that orthophosphate added as $Na_2HP^{32}O_4$ to respiring or fermenting yeast suspensions was rapidly resorbed and incorporated into ATP and other acid-labile fractions. During fermentation the specific activity of orthophosphate was higher than that of ATP, probably as a result of a heterogeneity in the intracellular orthophosphate. In respiring yeast, pyrophosphate was formed, the specific activity of which was very high when the cells were maintained from the start under aerobic conditions. Low specific activity was observed when respiration was preceded by a 30 to 60 min. period of fermentation. An acid-insoluble phosphate fraction was mobilized concurrently and is thought to be an intermediate in the pathway between ortho- and pyro-phosphate. It was observed that DPN undergoes a temporary resynthesis during the first five to six hours of respiration. Since there is no reason to suppose a difference in molecular structure of DPN in fermenting and respiring systems, it was suggested that the temporary resynthesis of DPN by respiring yeast is a secondary consequence of an enzymatic adaptation to aerobic metabolism. The prosthetic groups may become uncoupled from their proteins during this adaptation period, and assume their place in aerobic energy and phosphate transfer only after being reconjugated with the adaptive enzyme system. Presumably the accumulation of pyrophosphate occurs during this adaptation period.

Dagley, Dawes & Morrison (35) investigated the effect of aeration on the growth of *Aerobacter aerogenes* and *E. coli* with reference to the mechanism

of the Pasteur effect. They found that for anaerobic and semi-anaerobic cultures in a glucose, ammonium salt medium the linear relation between stationary population and concentration broke down at 8.6×10^{-3} *M* glucose and 1.3×10^{-3} *M* ammonium sulphate. No increase in crop resulted from further nutrient additions, and cell division ceased with substrates still available. Growth resumed upon gentle aeration but was delayed if the aeration was vigorous. Concomitantly there was an accumulation of pyruvate. Evidence was presented to suggest that this phenomenon was due to a resumption of fermentation with the production of pyruvate at a rate greater than its utilization during growth. The bearing of these observations on the Pasteur mechanism is duscussed.

Miscellaneous.—Jackins & Barker (36) reported a study of the fermentative patterns of the fusiform bacteria, a group for which little metabolic data are available. *Fusobacterium nucleatum* decomposed both amino acids and carbohydrate by a modified butyric acid type of fermentation, whereas *F. plautivincenti* produced a simple lactic fermentation of glucose, decomposed pyruvate to formate, acetate, and CO_2, and did not ferment amino acids.

An interesting type of fermentation by anaerobic micrococci was reported by Douglas (37). Glycine appears to be the principal substrate of *Micrococcus anaerobius* and *M. variabilis* and its decomposition is apparently similar to that found in *Diplococcus glycinophilus* (38), *i.e.*,

$$4CH_2NH_2COOH+2H_2O\rightarrow4NH_3+3CH_3COOH+2CO_2, \qquad 9.$$

when conducted under hydrogen. In an atmosphere of nitrogen *D. glycinophilus* produced hydrogen in addition to these products, but the micrococci showed no change.

The concept that inositol may be in equilibrium with glucose in biological systems was tested by Volk & Pennington (39). Employing a strain of *Aerobacter* capable of fermenting both glucose and inositol, they found a qualitative and a quantitative difference in the products of fermentation, suggesting that glucose is not an intermediate in inositol fermentation.

Racker (40) studied the mechanism of action of glyoxalase and demonstrated two enzymes, which he was able to purify, in baker's yeast. Glyoxalase I catalyzes the condensation of methylglyoxal and glutathione to an intermediate which is split by glyoxalase II to lactic acid and glutathione.

An interesting reserve carbohydrate other than glycogen or trehalose that is transiently formed during glucose fermentation by yeast cells was observed by Fales (41). The carbohydrate is insoluble in 30 per cent potassium hydroxide, its synthesis is blocked by azide, and it is synthesized and degraded at a more rapid rate than the alkali soluble reserve carbohydrate.

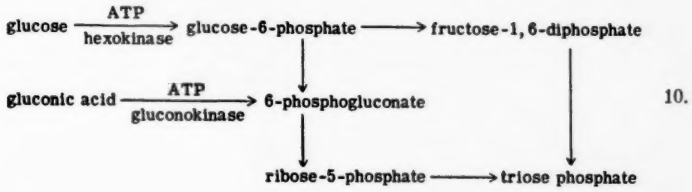
Convincing data on the inhibition of glucose fermentation by yeast juice by the addition of antibody to triosephosphate dehydrogenase were presented by Krebs & Wright (42). The antibody had no effect on the intact cell presumably because of the impermeability of the cell to the protein. A

similar study with yeast carboxylase was reported by Pasternak, Sevag & Miller (43).

The results of the effects of various ions, with particular emphasis on ion antagonisms, on the rate of glycolysis of bacterial cell suspensions were presented by Tsuyuki & MacLeod (44).

Oxidation

Oxidation of glucose-6-phosphate.—An alternative pathway of glucose-6-phosphate degradation, via direct oxidation (hexose monophosphate shunt) rather than by the well-recognized Meyerhof-Embden pathway, has been suggested by various researchers since 1938. However, this subject has remained obscure until recently. Contributions from Cohen's laboratory (45, 46, 47) have been extensive and have depended upon the development of chromatographic procedures for the identification of some of the intermediates of glucose-6-phosphate oxidation, as well as the use of pairs of adapted and nonadapted bacteria for specific fermentation analysis. They demonstrated 6-phosphogluconate as an enzymatic product of the oxidation of glucose-6-phosphate in extracts of yeast and *E. coli*, and obtained evidence for the formation of substances behaving like ribose-5-phosphate, arabinose-5-phosphate, and a new Bial-reactive phosphate, as well as glyceraldehyde-3-phosphate as products of 6-phosphogluconate oxidation. Hydrolysis of the phosphate esters revealed ribose and arabinose. Since the analysis of isolated pentoses revealed D-ribose and D-arabinose in amounts of approximately 25 and 10 per cent respectively of the total pentose present, the possible existence of hitherto undescribed compounds involved in 6-phosphogluconate degradation is suggested. The interrelationships between the oxidative pathway and the Meyerhof-Embden scheme are suggested by the following scheme (45):



10.

It will be of considerable interest and importance to learn the quantitative degree of glucose dissimilation in a given organism by each of these pathways.

Horecker & Smyrniotis (48) advanced our knowledge measurably by purifying a 6-phosphogluconic acid dehydrogenase from extracts of yeast which converts this substrate quantitatively to CO_2 and pentose phosphate which is not oxidized further. The pentose phosphates were identified on the basis of chromatographic and chemical properties and the formation of hy-

drazone derivatives as ribose-5-phosphate and the corresponding ketose, ribulose-5-phosphate [Horecker, Smyrniotis & Seegmiller (49)]. They presented the following clarification of the mechanism of phosphogluconic acid oxidation:

$$\begin{array}{l} \text{6-phosphogluconic acid} \xrightarrow{-2H} \text{[3-keto-6-phosphogluconic acid]} \xrightarrow{-CO_2} \\ \text{ribulose-5-phosphoric acid} \rightleftarrows \text{ribose-5-phosphoric acid} \end{array} \qquad 11.$$

Reports of studies with *Aerobacter* (50) and *Acetobacter* (51), while brief, do suggest the existence of a pathway for glucose degradation other than the Meyerhof-Embden system. Investigation of the relative utilization of labeled fructose-6-phosphate and fructose-1,6-diphosphate by *E. coli* led Roberts & Wolffe (52) to suggest that fructose-6-phosphate is oxidized directly to pentose phosphates.

Stokes & Campbell (53) reported that dried cells of *Pseudomonas aeruginosa* catalyze a nonphosphorylative conversion of glucose and gluconic acid to 2-ketogluconic acid which was the only compound detected at the end of the reaction. 2-Ketogluconate was not oxidized by these preparations. Entner & Stanier (54) studied glucose and gluconate oxidation by *Pseudomonas fluorescens* after growth on media containing either asparagine or glucose. The asparagine-grown cells were unadapted to the oxidation of gluconic and 2-ketogluconic acids but were immediately able to oxidize glucose. Under conditions (after ultraviolet irradiation) which prevented subsequent adaptation to gluconate and 2-ketogluconate it was found that these cells oxidized glucose with a quantitative accumulation of gluconate and without any evidence of glycolysis. On the other hand, the glucose-grown cells oxidized glucose and gluconate immediately, whereas 2-ketogluconate was oxidized slowly at the beginning but the rate was increased slightly thereafter. These observations were interpreted to suggest that glucose dissimilation proceeds largely via gluconic acid, although the possibility of glycolysis was not excluded. However, although 2-ketogluconate accumulated during the oxidation of glucose or gluconate, the manometric data showed that the oxidation of these substances could not proceed chiefly via 2-ketogluconate. Several possibilities were offered in explanation, but their data did not permit a decision.

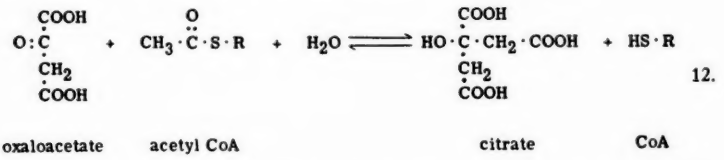
Terminal respiration.—Although the Krebs tricarboxylic acid cycle appears to be well established in animal tissues, its existence and role in microorganisms have been quite debatable. On the basis of work reported to date it would appear that the tricarboxylic acid cycle, in whole or in part, does function in some microorganisms, while a dicarboxylic acid cycle for the oxidation of C_2 fragments also exists, and is in some cases apparently more important than the tricarboxylic acid cycle. Associated with studies of these cycles in microorganisms are formidable problems indeed, not the least of which are difficulties in obtaining active cell-free preparations, lack of knowledge of some of the cofactors involved, the coexistence of a tricarboxylic and a dicarboxylic acid cycle in whole or in part, the difficulties involved in de-

grading the compounds associated with these cycles so that the specific activity of each carbon atom may be ascertained in tracer experiments, and the cycling and exchange reactions which occur in complex systems. The reader is referred to a competent and timely review of the terminal respiratory patterns in microorganisms [Ajl (55)].

The nature of the condensation reaction leading into the tricarboxylic acid cycle is somewhat controversial, and it may well be that more than one type of reaction exists. Thus for example, Umbreit, Smith & Oginsky (56) in a continuation of their studies on the action of streptomycin demonstrated citrate formation from oxaloacetate and pyruvate by *E. coli*. It was found that citrate formation from oxaloacetate and pyruvate was not inhibited by streptomycin in a streptomycin-sensitive strain, whereas the oxidation of a mixture of these substrates was inhibited. Also, a streptomycin-resistant strain, although incapable of appreciable oxidation of pyruvate and oxaloacetate, was found to form amounts of citrate comparable to those produced by the sensitive strain. These facts suggested that the condensation of acetate and oxaloacetate cannot be the sole or even the major pathway by which pyruvate enters the terminal respiratory system of this organism. Apparently the streptomycin-inhibited reaction does not proceed through citrate. They therefore postulated a pyruvate-oxaloacetate reaction not forming citrate, and it is this reaction that is inhibited by streptomycin. This conclusion is at variance with most contemporary studies in which it is thought that the condensation of acetate and oxaloacetate is the major pathway for the oxidation of pyruvate. On the basis of the streptomycin data the picture appears to be more complicated. These workers point out that there is no reason why both an acetate-oxaloacetate and a pyruvate-oxaloacetate reaction can not occur simultaneously, each competing with the other for available substrate.

In a series of four papers, Ochoa and his group have contributed richly to the general problem of the enzymatic synthesis of citric acid. Stern & Ochoa (57) demonstrated the synthesis of citrate from acetate, oxaloacetate, and ATP by soluble enzyme preparations from acetone-dried pigeon liver as well as by a mixture of soluble enzymes from extracts of *E. coli* and of a number of animal tissues or of yeast. The bacterial extracts supply two enzymes which are needed for the formation of "active acetate" while the tissue preparations contain condensing enzyme which catalyzes the condensation of "active acetate" and oxaloacetate to give citrate. Extracts from some bacteria, including *E. coli*, contain condensing enzyme. The enzymatic synthesis of citrate requires coenzyme A (Co A) and magnesium or manganese ions as cofactors. In the second paper, Ochoa, Stern & Schneider (58) reported the isolation of crystalline condensing enzyme from pig heart and presented quantitative data on the distribution of this enzyme in animal tissues and several microorganisms. It is interesting to note the absence of condensing enzyme in the anaerobe tested, *Clostridium butylicum*, and its presence in different concentrations in yeast, *Proteus vulgaris*, *Pseudomonas*

fluorescens, Azotobacter agile, and *Mycobacterium tuberculosis.* The reaction catalyzed by the condensing enzyme is formulated as: (59)



oxaloacetate acetyl CoA citrate CoA

12.

The fourth contribution by Korkes *et al.* (60) demonstrated that the dismutation of two molecules of pyruvate to acetyl phosphate, CO_2, and lactate in the presence of orthophosphate requires two enzyme fractions isolated from *E. coli,* transacetylase, lactic dehydrogenase, diphosphothiamine, DPN, and CoA. TPN was inactive. When oxaloacetate and condensing enzyme were substituted for orthophosphate and transacetylase, citrate was formed instead of acetyl phosphate, but there was no reaction in the absence of an acetyl acceptor system. They concluded that enzyme fractions A and B from *E. coli* catalyze a reaction between pyruvate, CoA, and DPN to form acetyl-CoA, CO_2, and reduced DPN.

The Ogston concept relating to the asymmetry of the citric acid-enzyme complex was studied by Carson, Mosbach & Phares (61) with *Aspergillus niger.* They utilized citrate synthesis from sucrose in the presence of sodium acetate-2-C^{14} and $C^{13}O_2$. The finding of wide divergence in citrate labeling between the primary carboxyls confirmed this concept in the case of a microbial system. Phares (62) and Mosbach, Phares & Carson (63) presented techniques which permit the determination of the isotopic concentration at each individual position of the carbon chain in the degradation of propionate, acetate, citrate, α-ketoglutarate, and glutamate.

Biosynthesis and reactions of organic acids.—Kornberg & Pricer (64) purified two distinct enzyme systems from baker's and brewer's yeast catalyzing the oxidative decarboxylation of D-isocitrate to α-ketoglutarate. One system was found to be TPN specific and capable of catalyzing the decarboxylation of oxalosuccinate, the reductive carboxylation of α-ketoglutarate, and the reduction of oxalosuccinate, thus resembling the system in animal tissues. The other system was DPN specific; it failed to catalyze the reductive carboxylation of α-ketoglutarate, and oxalosuccinate does not appear to be an intermediate. In contrast to the former system it showed a specific and absolute requirement for adenosine-5-phosphate.

The adaptive enzyme catalyzing L-malic acid $\overset{DPN;Mn^{++}}{\rightleftharpoons}$ lactic acid $+CO_2$ has been purified further by Kaufman, Korkes & del Campillo (65) and has been shown to form exclusively L-lactate in contrast to previous preparations which formed DL-lactate from L-malate, presumably as a result of contamination with a D- and an L-lactic dehydrogenase. With the use of L-malate labeled in both carboxyl groups with C^{14}, it was demonstrated that

pyruvate is not a free intermediate in the over-all reaction catalyzed by the purified enzyme.

McManus (66) investigated CO_2 fixation by lysed suspensions and extracts of *Micrococcus lysodeikticus*. The examination of the incorporation of labeled CO_2 in oxaloacetate and malate indicated a much higher isotope activity in oxaloacetate with little or no change in the relative distribution of isotope on the addition of TPN or DPN. There was no evidence that a reaction other than one involving oxaloacetate carboxylase was taking place. The results indicated the nonparticipation of the malic-TPN system in CO_2 fixation by this organism and the direct incorporation of CO_2 by the Wood-Werkman reaction. Kaltenbach & Kalnitsky (67, 68) demonstrated the formation of large amounts of oxaloacetate from pyruvate and bicarbonate by enzyme extracts of *E. coli* and *Proteus morganii* under anaerobic conditions. From the results of isotope experiments the nonparticipation of the malic-TPN system was suggested. The optimum conditions for the carboxylation reaction in addition to the enzyme appear to be: (*a*) pyruvate, 0.07 to 0.08 *M*, (*b*) bicarbonate, 0.19 *M*, (*c*) pH 8.1, (*d*) inorganic phosphate, 0.09 *M*, (*e*) Mg^{++}, 0.07 *M*, and (*f*) biotin, approximately 3.9×10^{-5} *M*.

An interesting study by Carson *et al.* (69) resulted in the demonstration of an oxidative mechanism of lactic acid formation from ethanol via a 2 C_2 condensation in strain MX of a *Rhizopus*. A glycolytic formation of lactate apparently occurs concomitantly with the oxidative formation.

A hitherto unknown type of photosynthesis was observed in *Chlorobium thiosulphatophilum* by Larsen (70). Resting cells illuminated in the presence of propionic acid and CO_2 formed succinate as a major product. No reaction was demonstrable in the dark. A C_3-C_1 condensation was suggested since the succinate was synthesized from CO_2 and propionate carbon.

The nonidentity of cytochrome-*b* and succinic dehydrogenase is claimed by Johns (71) on the basis of finding succinic acid dehydrogenase activity in *Veillonella gazogenes* without detectable cytochrome bands. Other lines of evidence with animal tissue enzyme preparations also suggest the nonidentity of the two [Tsou (72)].

Fatty acid oxidation.—Silliker & Rittenberg (73) investigated the ability of several microorganisms to oxidize capric and pelargonic acids with the finding that the oxidation of these saturated fatty acids was not uncommon among aerobic bacteria and that the enzymes concerned were in most instances constitutive. In a subsequent report (74) they applied the technique of simultaneous adaptation to a study of the mechanism of fatty acid oxidation by *Serratia marcescens*. On the basis of their results they postulated that a single enzyme system catalyzes the degradation of all fatty acids oxidized by this organism and that the individual enzymes function in a repeating sequence. They claim further that their data are consistent with either a multiple alternate oxidation or a modified beta oxidation of fatty acids.

Butyrate and vinyl acetate oxidation to acetoacetate by enzyme preparations from *Clostridium kluyveri* in the absence of inorganic phosphate was

reported by Kennedy & Barker (75). Several C_4 compounds were excluded as possible intermediates in butyrate oxidation. The significance of their results in relation to the mechanism of synthesis and oxidation of butyrate is discussed in detail.

Oxidative assimilation.—A study of oxidative assimilation in *Pseudomonas saccharophila* by use of C^{14}-labeled substrates was made by Wiame & Doudoroff (76). Attempts were made to determine the metabolic fate of individual carbon atoms of the substrates during their oxidation by resting cells and to ascertain the effect of oxidation of exogenous substrates on endogenous respiration. They observed a negligible reduction of CO_2 to cell material during oxidative assimilation of organic compounds. The endogenous respiration of the cells was inhibited during the oxidation of added substrates, except in the presence of dinitrophenol. With acetate as substrate it was determined that both carbon atoms are assimilated, the methyl carbon being favored especially in the presence of a nitrogen source. The carboxyl carbon of lactate was almost completely oxidized with the remaining C_2 fragment being used almost entirely for synthetic reactions, while with succinate both carboxyl carbons were oxidized to CO_2, and the methylene carbons were largely assimilated. They interpreted these findings to support the view that C_2 fragments of the nature of "active acetate" are fundamental building blocks in the oxidative assimilation of this organism. It is pertinent that Fraser & Tolbert (77) found an almost identical pattern of assimilation of carbon atoms of C^{14}-labeled lactate in growing cultures of *E. coli*. The carboxyl carbon was largely but not exclusively converted to CO_2. The alpha and beta carbons were utilized primarily in synthetic reactions. However, some were converted to CO_2 and an appreciable amount of C_2 fragment left from the decarboxylation of lactate was excreted into the medium.

Nitrogen Metabolism

Nitrogen fixation.—Linstrom, Lewis & Pinsky (78) have extended the list of organisms capable of utilizing atmospheric nitrogen by demonstrating, with suitable techniques, this ability in five members of the family Athiorhodaceae, 19 strains of rhodopseudomonads, and *Rhodomicrobium vannielii*. However, the possibility that all organisms containing the enzyme hydrogenase may possess the ability to fix N_2 seems not necessarily true since *E. coli* and *Hydrogenomonas*, both of which have active hydrogenases, were not capable of fixing N_2 under the conditions tested. N_2 fixation by several species of the genus *Desulfovibrio* has also been demonstrated [Sisler & ZoBell (79)]. It is possible that the ability to fix N_2 may depend, as with many other physiological activities, on the conditions of cultivation.

Wilson and co-workers have obtained further evidence on the role of ammonia as an intermediate in N_2 fixation. With growing cultures of *Clostridium pasteurianum* it was found that only ammonia of several nitrogen compounds tested was used at a rate and an extent suggestive of a true intermediate [Rosenblum & Wilson (80)]. These results are in agreement with those re-

ported previously by this group employing *Azotobacter*. However, with *A. vinelandii* direct isolation of the intermediate has not been possible, presumably because ammonia is metabolized so rapidly by this organism. In a more recent report, Zelitch *et al.* (81) found that when *C. pasteurianum* cells actively fixing molecular nitrogen were supplied N_2^{15} for a short time, free ammonia with an extremely high N^{15} concentration could be isolated from the supernatant medium. Somewhat lower concentrations were found in the amide fraction. They were also able to demonstrate the accumulation of N^{15} in the glutamic acid of the cells (82). These several papers demonstrate more conclusively that ammonia is a key intermediate in nitrogen fixation by *Clostridium*, and suggest a marked similarity in the mechanism of nitrogen fixation by aerobes and anaerobes.

Amino acid assimilation.—Gale and associates have continued their studies on the assimilation of amino acids by bacteria and have reported their findings in a series of papers. It was found that sodium azide and 2,4-dinitrophenol inhibited the accumulation of free glutamate in *Micrococcus pyogenes* cells while at the same concentrations they increased the amount of glutamate in *Streptococcus faecalis* cells [Gale (83)]. The effect was correlated in *S. faecalis* with the fact that these substances inhibit the internal metabolism of glutamic acid. Working with *M. pyogenes* he found (84) that whether glutamate accumulates in the free state within the cell or enters into combination within the cell depends on the balance between glutamic acid and the other amino acids in the medium. Thus for example, when the external concentration of glutamate was raised to 100 times that of the other amino acids in the menstrum, some accumulation of free glutamate occurred, but the formation of cellular combined glutamate was suppressed. The action of various inhibitors on the accumulation of free glutamate and the formation of combined glutamate was reported by Gale & Paine (85). Of the two processes (*a*) accumulation of free glutamate and (*b*) the formation of cellular combined glutamate, interesting differences were noted. Sodium azide and 2,4-dinitrophenol were equally effective against both, 8-hydroxyquinoline was more effective against (*a*) than (*b*), whereas aureomycin and chloramphenicol were more effective against (*b*) than (*a*). The concentrations employed were ineffective in inhibiting fermentation by the cells. Streptomycin inhibited (*b*) but at concentrations higher than those required to prevent growth, whereas a close correlation was found between the inhibitory action of chloramphenicol on (*b*) and its action as a growth inhibitor. While penicillin had no effect on these processes in cell suspensions, the addition of penicillin to the medium 30 to 60 min. before harvesting resulted in complete inhibition of both (*a*) and (*b*). Interesting results were obtained on extracellular peptide formation by *M. pyogenes* which apparently accompanied a decrease in the rate of accumulation of internal free glutamate [Gale & van Halteren (86)]. Glucose was essential for peptide formation just as it is for the assimilation of glutamate.

Amino acid synthesis.—Although the recent demonstration of a wide

variety of transaminases makes possible a general mode of amino acid synthesis from aspartate and glutamate, it is quite evident that unique biosynthetic reactions for individual amino acids exist. The study of these systems has progressed rapidly with the use of biochemical mutant strains of microorganisms.

α-Aminoadipic acid has been identified as a precursor for lysine synthesis by *Neurospora crassa* by Windsor (87), thus confirming the postulate of Mitchell & Houlahan (88).

Adelberg, Bonner & Tatum (89) isolated and characterized α,β-dihydroxy-β-ethylbutyric acid from *N. crassa* as a precursor of isoleucine. The mutant required both isoleucine and valine for growth, presumably because of the accumulation of an isoleucine precursor which inhibits the conversion of some analogous intermediate in valine synthesis. Studies with an isoleucine-requiring strain of *E. coli* showed also that α,β-dihydroxy-β-ethylbutyric acid was a normal intermediate in the biosynthesis of isoleucine. The relationship between isoleucine and valine is suggested further by studies on the derivation of the carbon atoms of both amino acids [Tatum & Adelberg (90)]. On the basis of their data with C^{14}-labeled acetate they presented the following general scheme: Two molecules of acetate couple to form an unknown C_4 intermediate which in turn couples with the methyl group of another molecule of acetate to form a C_6 dicarboxylic acid; this undergoes reduction of the side chain carboxyl to give the carbon skeleton of isoleucine, while decarboxylation yields the valine configuration. Umbarger & Adelberg (91) reinvestigated some of their studies on the basis that the ketoisoleucine employed previously was now identified as α-ketobutyric acid. Their data support a biosynthetic scheme in which the α,β-dihydroxy acids corresponding to isoleucine and valine are converted to their respective alpha-keto acids prior to amination.

Methionine-threonine-homoserine interrelationships are supported further by the studies of Fling & Horowitz (92). They observed that extracts of a methionineless mutant strain of *N. crassa* contained two substances, one active for a threonineless mutant and one for a homoserineless mutant. The first substance was identified as L-threonine and the second is probably L-homoserine which is the first report of the presence of this substance in a biological system. A metabolic relation between homoserine and canavanine is suggested by Teas (93).

An interesting study by Wright (94) showed that a *Neurospora* mutant requiring glycine or serine can grow on glyoxylic or glycolic acid, although not to the same degree. Glycine was more effective than serine and glyoxylic acid, whereas glycolic acid was as effective as glycine. They interpreted these results to suggest that glycolic acid can be oxidized to glyoxylic acid which can then be aminated to glycine. Further work is obviously required to determine whether such an amination can take place.

By use of mutant strains of *E. coli*, Davis (95) has contributed richly to our knowledge of the biosynthesis of aromatic compounds. Mutants were

obtained with a multiple requirement for tyrosine, phenylalanine, tryptophan, and *p*-aminobenzoic acid (PAB), and a relative requirement for *p*-hydroxybenzoic acid. It was found that shikimic acid (a trihydroxycyclohexenecarboxylic acid) substitutes for the quintuple requirement apparently by functioning as an early intermediate in aromatic synthesis. This conclusion was supported by demonstrating the accumulation of shikimic acid in the culture filtrates of other mutants with aromatic requirements. Still other mutants accumulate an unknown thermolabile precursor of shikimic acid. No other available aromatic or hydroaromatic compound was found capable of satisfying the multiple aromatic requirement. Of many compounds tested as possible intermediates between shikimic acid and the aromatic amino acids, only the corresponding α-keto acids substituted for phenylalanine and tyrosine, and indole and anthranilic acid for tryptophan.

Reactions of amino acids.—Stanier, Hayaishi & Tsuchida (96) obtained data which confirm the existence of aromatic and quinoline pathways for the bacterial oxidation of tryptophan by *Pseudomonas* species. With one exception the initial reactions were common to all strains studied and involved attack on the five-membered ring of the indole nucleus (tryptophan→formylkynurenine→kynurenine). Among those strains that oxidize tryptophan completely, there appear to be two distinct pathways beyond kynurenine. Some degrade the kynurenine to anthranilic acid and then to catechol (aromatic pathway), while others degrade it to kynurenic acid (quinoline pathway). Enzymatic studies on cell-free extracts of strains which employ the aromatic pathway showed the following transformations:

$$\text{catechol} + O_2 \rightarrow \textit{cis-cis}\text{-muconic acid} \xrightarrow{+H_2O} \beta\text{-ketoadipic acid} \qquad 13.$$

It is interesting to note that extracts prepared from cells not adapted to tryptophan do not contain detectable amounts of these enzymes. This demonstrates that adaptation to metabolize tryptophan involves synthesis by the cells of the necessary enzyme systems [Hayaishi & Stanier (97)]. The later intermediates in the quinoline pathway have not yet been detected by these workers. Some aspects of aromatic fission type of metabolism in bacteria are reviewed briefly by Evans *et al.* (98).

The tryptophanase reaction (tryptophan→indole+pyruvate+ammonia) was studied in *E. coli* by use of 5-methyltryptophan as the substrate [Beerstecher & Edmonds (99)]. On the basis of their results they suggested that indole is required in catalytic quantities as a cofactor, and postulated a new role for pyridoxal phosphate which is generally considered to be the coenzyme of this reaction. Although they admit that the use of an analogue as a substrate may involve a different reaction mechanism than is usual, they nevertheless discount this possibility in the present case. It would appear that these data might well be interpreted with caution, at least until similar studies are made with tryptophan.

Homocysteine is metabolized by cell-free extracts of *Proteus morganii*

to α-ketobutyrate, ammonia, and H_2S, while cysteine yields pyruvate, ammonia, and H_2S in the typical desulfhydrase system. Pyridoxal phosphate functions as a cofactor in this system [Kallio (100)].

The well-known adverse effect of the presence of glucose in growth media on the production of deaminases by bacteria (101) has been reinvestigated by Boyd & Lichstein (102). Their data suggest that the glucose effect is not primarily on apoenzyme production but apparently on the coenzyme concentration. It may be that glucose somehow prevents the formation of the coenzyme or accelerates its destruction.

A spectrophotometric method for studying the kinetics of transaminase systems has been presented by Cammarata & Cohen (103) who also studied some of the properties of the glutamic-oxaloacetic transaminase (104).

Evidence for the decarboxylation of L-aspartic acid at the beta carboxyl group to yield α-alanine has been obtained by Meister, Sober & Tice (105) in extracts from *Clostridium welchii*. Decarboxylation of aspartate to yield β-alanine had been reported previously by Billen & Lichstein (106).

An interesting enzyme system apparently widely distributed in microorganisms and other tissues which catalyzes the exchange of the amide group of asparagine and glutamine with hydroxylamine or isotopic ammonia has been reported by Schou, Grossowicz & Waelsch (107). They investigated the effects of several agents on the inhibition and activation of glutamohydroxamic and aspartohydroxamic acids in cell-free extracts of *P. vulgaris*.

Thayer & Horowitz (108) studied the L-amino acid oxidase of *N. crassa*. The production of the enzyme was greatly increased by cultivation on media containing a marginal supply of biotin and amino acids. It was found that the enzyme is three to six times more active in oxygen than in air, and that the pH optimum varies with the amino acid used as substrate. Enzyme inhibition was noted at high substrate concentration, and the simultaneous addition of two amino acids resulted in an interference effect. Burton (109) reported similar effects of biotin and amino acids on the production of this enzyme in both *N. crassa* and *N. sitophila*. A broad maxima between 6 and 9.5 was found, and flavin-adenine-dinucleotide (FAD) appeared to be the prosthetic group.

The discovery of an alanine racemase in *S. faecalis* capable of converting both D- and L-alanine to a racemic mixture [Wood & Gunsalus (110)] is an important contribution which will aid materially in a better understanding of the biological significance of the D-amino acids. Pyridoxal phosphate was required as a coenzyme for the alanine racemase while pyridoxamine phosphate was without activity. The optimum pH of the purified enzyme is greater than 8, and the enzyme appears to be specific for alanine. It is tempting to assume that other amino acid racemases will be discovered.

Nucleic acids and derivatives.—E. coli contains enzymes which split desoxyribose nucleosides to yield the free base and a desoxyribose ester, probably desoxyribose-1-phosphate. These enzymes may also catalyze the synthesis of nucleosides [Manson & Lampen (111)]. The cleavage of adenosine,

cytidine, and xanthosine by *L. pentosus* was studied in resting cells and extracts by Wang & Lampen (112). Resting cells in arsenate buffer split uridine but not thymidine, while cell-free extracts contained nucleosidases acting on adenosine, cytidine, and xanthosine. Evidence was presented for a direct split of adenosine to yield adenine and of cytidine to yield cytosine. Nucleosidases, previously found in other tissues, acting on guanosine, inosine, and uridine were found in this organism. The nucleosidases of *L. pentosus* cleave the ribosides of hypoxanthine, cytosine, and uracil rapidly and the corresponding desoxyribosides very slowly if at all.

Kornberg & Pricer (113) have contributed to our understanding of the synthesis of adenosine-5-phosphate by establishing the conversion of adenosine to adenosine-5-phosphate as a simple and direct transfer from ATP. They obtained a partially purified enzyme from autolysates of brewers' yeast which catalyzes the direct phosphorylation of adenosine and of 2,6-diaminopurine riboside (2-aminoadenosine) by ATP:

$$\text{adenosine} + \text{ATP} \rightarrow \text{adenosine-5-phosphate} + \text{ADP} \qquad 14$$

$$\text{2-aminoadenosine} + \text{ATP} \rightarrow \text{2-aminoadenosine-5-phosphate} + \text{ADP} \qquad 15$$

This adenosine phosphokinase is specific for adenosine and 2-aminoadenosine among 17 nucleosides tested, and the transfer of phosphate is limited to the 5 carbon of the nucleoside.

A diphosphopyridine nucleotidase from *N. crassa* has been studied by Kaplan, Colowick & Nason (114) and found to be similar to the enzyme isolated from brain tissue since it attacked the nicotinamide-ribose linkage of DPN. The *Neurospora* enzyme apparently differs from the brain enzyme in the lack of inhibition by nicotinamide and the wide pH of activity in contrast to the sharp pH optimum of the latter.

Friedman & Gots (115) demonstrated the deamination of isoguanine (6-amino-2-hydroxypurine) to xanthine in suspensions of *E. coli*. Balis & Brown (116) reported that *Lactobacillus casei* readily transforms guanine to adenine, whereas yeast apparently cannot but is able to catalyze the reverse reaction (adenine to guanine) [Kerr, Seraidarian & Brown (117)]. This fact was ascertained since adenine was utilized for the formation of ribonucleic acid (RNA) adenine and guanine by the yeast, while guanine, which was utilized equally well, was incorporated only into RNA guanine. It was also found that the presence of preformed guanine sufficient for the biosynthesis of RNA guanine inhibited the transformation of adenine to guanine.

The fermentation of purines by two strains of *Micrococcus lactilyticus* was investigated by Whiteley & Douglas (118). One of the most interesting findings was that one strain decomposed hypoxanthine to equivalent amounts of xanthine and hydrogen, thus representing a hitherto unknown method of hydrogen production by bacteria.

Evidence for the incorporation of the glycine molecule into adenine and guanine was obtained with *A. aerogenes* using doubly labeled glycine as a substrate [Sutton, Schlenk & Werkman (119)].

Functions and Metabolism of Nutritional Factors

Trace metals.—Nossal (120) reported the activation of the L-malate decarboxylase system of *L. arabinosus* by potassium ions, the effectiveness increasing up to a concentration of 0.1 *M*. Potassium could be replaced by rubiduim and possibly caesium, but not by lithium, sodium, or ammonium ions. Black (121) isolated a soluble enzyme from bakers' yeast which catalyzes the reduction of pyridine nucleotides by several aldehydes. Potassium ions were required for activity, but rubidium or ammonium ions could be substituted. Lithium, sodium, and caesium were inhibitory. Meyerhof & Kaplan (33) in a study of factors influencing the rate of fermentation of yeast found that fermentation was completely lacking in the absence of either potassium or ammonium ions and of magnesium.

Growth studies with *Aspergillus niger* revealed that traces of zinc, iron, copper, and manganese were required for high yields of citric acid [Tomlinson, Campbell & Trussell (122)]. Omission of zinc from the growth medium caused a 96 per cent decrease in citric acid production, while deletions of iron, manganese, or copper resulted in a 91 per cent, 44 per cent, and 76 per cent decrease respectively, Chesters & Rolinson (123) noted that a partial deficiency in zinc, iron, copper, manganese, magnesium, phosphorus, or potassium resulted in the production of larger quantities of acid per unit of glucose utilized than formed in normal cultures of *A. niger*.

Changes in the enzymatic constitution in mats of zinc deficient *Neurospora* were investigated by Nason, Kaplan & Colowick (124). Alcohol dehydrogenase was absent, and the enzyme which condenses indole and serine to form tryptophan was greatly reduced in amount. Fumarase was not influenced by the deficiency, while a 10 to 20-fold rise in the concentration of diphosphopyridine nucleotidase was noted. The addition of zinc had no direct effect on the enzymes studied. Calcium deficiency produced changes in the enzyme concentrations which were similar but not as marked as the zinc deficiency, whereas magnesium, manganese, and iron deficiencies had no effect on the level of these enzymes. They concluded that zinc deficiency results in a failure to synthesize enzyme proteins in the normal manner as well as in a decreased net protein synthesis.

B vitamins.—Pyridoxal phosphate is required for several reactions of amino acids in addition to those already reported. Restoration of desulfhydrase activity by pyridoxal phosphate after inactivation by aging and dialysis was reported by Kallio (100). Delwiche (125) confirmed the role of this vitamin in partially resolved cysteine desulfhydrase obtained from another organism, but in addition pointed out that other agents, namely biotin, adenosine-5-phosphate, and α-ketoglutarate stimulate this system. Further work is required before these results are completely compatible. It appears possible that a pyridoxal phosphate-biotin relationship exists. Excellent data on the coracemase activity of pyridoxal phosphate were given by Wood & Gunsalus (110). A role for pyridoxal phosphate in the decarboxylation of aspartate to α-alanine was suggested by Meister, Sober & Tice

(105). The activation of this enzyme by α-keto acids noted by these workers was explained on the basis of codecarboxylase formation by a transamination reaction between pyridoxamine phosphate and pyruvate. It would appear that this could be checked experimentally. During studies on the fractionation of the glutamic-oxaloacetic transaminase, Cammarata & Cohen (104) were unable to reactivate fully the resolved enzyme by additions of synthetic pyridoxal phosphate. Possible explanations offered were: (*a*) a metal requirement in addition to that for pyridoxal phosphate, (*b*) a difference between synthetic pyridoxal phosphate and the "natural" coenzyme, and (*c*) denaturation of the resolved enzyme, thus preventing complete recombination with coenzyme. It will be interesting to observe the resolution of this problem.

On the basis of N^{15} experiments and the use of mutant strains of *Neurospora*, Yanofsky & Bonner (126) obtained excellent evidence to show that the sole precursor of the pyridine nitrogen of nicotinic acid is the nitrogen of the amino group of 3-hydroxyanthranilic acid.

A most interesting and important function for coenzyme A (Co A) was reported by Stadtman, Novelli & Lipmann (127). They found that Co A is required for the catalysis of interchange of acetyl-bound and inorganic phosphate, and for the arsenolysis of acetyl phosphate by a phospho-transacetylation enzyme present in extracts of *C. kluyveri* and other bacteria. The evidence presented substantiates the assumption that an acetyl~Co A exists either free or combined to a protein, and the conclusion suggested was that "active acetate" is actually acetyl~Co A which may be derived by a variety of enzyme reactions from a variety of acetyl-donating molecules. In turn, acetyl~Co A may donate its acetyl residue to a variety of acceptor systems. On the basis of growth studies with strains of yeast and lactic acid bacteria, Craig & Snell (128) presented a scheme showing the interrelationships of some physiologically important derivatives of pantothenic acid. Streptomycin exerts a marked inhibitory effect on the growth of *Saccharomyces fragilis* when this organism is grown in a synthetic medium containing β-alanine or β-alanine plus pantoyl lactone as precursors for pantothenic acid [Lichstein & Gilfillan (129)]. In contrast, no effect on growth was obtained when streptomycin was incorporated into media containing preformed pantothenate. These data were interpreted to suggest that streptomycin inhibits the synthesis of pantothenic acid by preventing the coupling of β-alanine and pantoyl lactone to form pantothenate. If this interpretation is correct, it may help to explain some of the other reported actions of this antibiotic.

The involvement of biotin in the synthesis of proteins is suggested by the work of Thayer & Horowitz (108) and of Burton (109) on the L-amino acid oxidase of *Neurospora*. Likewise, Nason, Kaplan & Colowick (124) observed that biotin deficient *Neurospora* exhibit a loss of alcohol dehydrogenase activity and a pronounced increase in diphosphopyridine nucleotidase, results which are similar to those observed when there is a zinc deficiency. Biotin

involvement in the cysteine desulfhydrase system [Delwiche (125)] has been discussed. The function of this vitamin in the deaminase systems has been studied by Boyd & Lichstein (102). Resolved cells were obtained by growth in a glucose medium, and deaminase activity could be restored readily by the addition of biotin, adenosine-5-phosphate, or yeast extract to the cell suspensions. Moreover, the resolving effect of glucose could be prevented during growth if high concentrations of preformed coenzyme or coenzyme precursors were added to the growth medium. Biotin does not appear to be involved in apoenzyme production in the deaminase systems. The role of biotin in the Wood-Werkman reaction has been reinvestigated by Kaltenbach & Kalnitsky (68) in cell-free preparations derived from *P. morganii*. They found that the *in vitro* addition of biotin to extracts of cells harvested from a vitamin-deficient medium resulted in marked stimulation of oxaloacetate formation from pyruvate and bicarbonate, but that the activity was not brought up to normal levels. This suggested that some other factor is necessary either for the formation of the enzyme or as a component of the enzyme system. The addition of both biotin and magnesium to extracts from biotin deficient cells gave better stimulation than either agent alone. In some experiments, however, biotin had little effect. They concluded that biotin is a necessary cofactor in this system. The functions of biotin in enzyme systems have been reviewed recently [Lichstein (130)].

The participation of PAB in the synthesis of vitamin B_{12} is suggested by the sparing effect of B_{12} on the PAB requirement of mutant strains of *E. coli* [Davis (131)]. It was suggested that the relation of PAB to methionine synthesis appears to depend on the role of PAB in the formation of B_{12} which in turn takes part in the formation of methionine from homocysteine. These implications may help to explain the results of Ben-Ishai, Volcani & Bergmann (132). The relationship of PAB to another newly discovered vitamin, *p*-hydroxybenzoic acid is discussed by Davis (133).

The influence of vitamin B_{12} on oxidation by a mutant strain of *E. coli* which requires methionine or B_{12} for growth was studied by Oginsky *et al.* (134). It was observed that this vitamin increases markedly the rate of oxidation of a variety of substrates by resting cell suspensions. Although the mechanism of stimulation was not elucidated, some indication that B_{12} may be concerned in the synthesis of porphyrins was obtained. Further study is required to establish this relationship. The role of B_{12} in metabolic processes was reviewed by Jukes & Stokstad (135).

A noteworthy contribution to our understanding of the metabolism of riboflavin is the isolation of an enzyme, flavokinase, from brewers' yeast which catalyzes the phosphorylation of this vitamin [Kearney & Englard (136)].

$$\text{riboflavin} + \text{ATP} \rightarrow \text{riboflavin-5-phosphate} + \text{ADP} \qquad \textbf{16.}$$

A possible role of riboflavin in the adaptive peroxidation by *S. faecalis* is discussed by Seeley & Rio-Estrada (137).

Growth studies with *Candida lipsolytica* suggested that thiamine is required for lipase production [Peters & Nelson (138)]. The functions of B vitamins in the biosynthesis of purines and pyrimidines was reviewed by Shive (139), and antimetabolites of nucleic acid metabolism are discussed by Wright (140).

Other factors.—A metabolic function for oleic acid which helps to explain the vitamin-like activity of certain fatty acids was reported by Lichstein & Boyd (141). Employing a biotinless mutant strain of *E. coli* they found an increased rate of fermentation of glucose, pyruvate, and formate by cells grown in the presence of oleate as compared to the same cells grown with biotin. These observations were confirmed with other wild strains of microorganisms. An examination of formate metabolism revealed that the biotin-grown cells were deficient in both formic dehydrogenase and formic hydrogenlyase, while the oleate-grown cells exhibited excellent activity. The fact that formic hydrogenlyase activity of resting cell suspensions could be stimulated by the addition of oleic acid suggested that this fatty acid, or some substance readily derived from it, functions as a cofactor in this enzyme system.

The pyruvate oxidase factor was identified as α-lipoic acid, which was isolated in crystalline form by Reed *et al.* (142). According to these investigators, α-lipoic acid also represents one of several acetate-replacing factors for lactobacilli and is the first member of a new family of B vitamins. This substance is very soluble in organic solvents but only sparingly soluble in water. In further communications (143, 144) extraction procedures, distribution, purification, and properties of this and other acetate-replacing factors were reported. An interesting relationship between the pyruvate oxidase factor and CO_2 is suggested by replacement of this factor by CO_2 [Lytle, Zulick & O'Kane (145)].

Enzymes

Polysaccharide synthesis.—In an excellent study, Hehre (146) demonstrated the biosynthesis of dextran from certain dextrins by soluble enzyme preparations from *Acetobacter capsulatum*. This represents the first time this type of polysaccharide has been synthesized from a source other than sucrose. The conversion of dextrins to dextran brings polysaccharides of the starch-glycogen and dextran classes into the closest biological relationship known to exist between any two polysaccharide classes. The enzyme system, dextran-dextrinase, attacks the products of partial hydrolysis of amylose, amylopectin, and glycogen when hydrolysis was carried out by acid or by salivary amylase. Dextran formation could not, however, be demonstrated from these substances before hydrolysis or from the products of their partial hydrolysis by β-amylase. Cycloheptaamylose was inert but both amyloheptaose and sodium amyloheptaoate were active as substrates. Quantitative study of the course of action of the enzyme revealed an exact correspondence between dextrin utilized and dextran formed, and also revealed that

the enzyme is able to catalyze dextran synthesis from molecules at least as small as amylotetraose. The mode of action of the enzyme system, whereby chains of 1,4-linked glucose units are converted to new chains of 1,6-linked units, appears to be fundamentally similar to that of other polysaccharide-synthesizing enzymes. The mechanism was pictured as the repetition of a single basic step, namely, the transfer of a glucosyl group from a terminal position in a dextrin molecule to a terminal position in a growing dextran molecule. The reaction represents the first known instance of the conversion of one polymeric material into another, in which essentially every linkage is affected.

Thermophilic enzymes.—Resolution of the problem of the heat resistance of thermophilic microorganisms is closer now because of the work of the Nebraska group with thermophile No. 2184. In earlier papers, Militzer *et al.* (147, 148) reported that malic dehydrogenase and cytochrome oxidase in extracts from this thermophile were stable at 65°C., a temperature that inactivated these enzymes from a mesophilic bacterium and from animal tissues. Since these enzymes were concentrated in an insoluble fraction called the "red fraction," and since the close association of protein in this fraction might account for the stability of the enzymes therein, they decided to study an enzyme that was not bound to this fraction. Apyrase was chosen [Militzer, Tuttle & Georgi (149)]. It was found that this enzyme possessed heat stability at 65°C. when extracted from the thermophile, while apyrase from a mesophilic organism did not have the resistance exhibited by the thermophilic enzyme. These results with an enzyme system divorced from the structure of the cell and from the packing effect that might occur in the insoluble particulate fraction certainly support the explanation which holds that the enzymes and proteins of thermophilic microorganisms possess a heat stability not shared by mesophiles. The absolute demonstration that the heat stability is inherent in the protein structure itself must await crystallization of such an enzyme. Additional evidence in support of the heat stability of enzymes from thermophiles comes from the work of Stark & Tetrault (150) on *Bacillus stearothermophilus.* They obtained a cell-free enzyme system (starch saccharifying enzymes) from the medium at 70°C. and tested it at 90°C. Although the identity of the enzymes was not elucidated, the heat stability was demonstrated clearly.

Enzymes in cytoplasmic granules.—The existence in certain bacterial species of intracellular granules containing a variety of enzymes, some organized in systems as in the mitochondria of animal cells, seems reasonably established on the basis of the work of Georgi *et al.* (151). They separated an insoluble "red fraction" by centrifugation from the lysate of a thermophilic bacterium which had been treated with lysozyme. The particulate nature of the fraction was demonstrated by microscopy, and enzyme activity was determined directly by suitable techniques. Their data demonstrate convincingly that these particles are the site of considerable enzyme ac-

tivity. Less direct evidence based on *in vivo* color reactions in other microorganisms is offered by Mudd *et al.* (152).

LITERATURE CITED

1. Gibbs, M., and DeMoss, R. D., *Federation Proc.*, **10,** 189 (1951)
2. DeMoss, R. D., Bard, R. C., and Gunsalus, I. C., *J. Bact.*, **62,** 499–511 (1951)
3. Gibbs, M., and DeMoss, R. D., *Arch. Biochem. Biophys.*, **34,** 478–79 (1951)
4. Rowatt, E., *Biochem. J.*, **49,** 453–62 (1951)
5. Nossal, P. M., *Biochem. J.*, **49,** xliv (1951)
6. Dolin, M. I., and Gunsalus, I. C., *J. Bact.*, **62,** 199–214 (1951)
7. Juni, E., *Federation Proc.*, **9,** 396 (1950)
8. Kohlmiller, E. F., Jr., and Gest, H., *J. Bact.*, **61,** 269–82 (1951)
9. Johns, A. T., *J. Gen. Microbiol.*, **5,** 326–36 (1951)
10. Barban, S., and Ajl, S. J., *J. Biol. Chem.*, **192,** 63–72 (1951)
11. Stadtman, T. C., and Barker, H. A., *J. Bact.*, **62,** 269–80 (1951)
12. Strecker, H. J., *J. Biol. Chem.*, **189,** 815–30 (1951)
13. Billen, D., *J. Bact.*, **62,** 793–97 (1951)
14. Billen, D., and Lichstein, H. C., *J. Bact.*, **61,** 515–22 (1951)
15. Stadtman, T. C., and Barker, H. A., *J. Bact.*, **61,** 67–80 (1951)
16. Stadtman, T. C., and Barker, H. A., *J. Bact.*, **61,** 81–86 (1951)
17. Garner, R. L., and Grannis, G. F., *Science*, **114,** 501–2 (1951)
18. Caputto, R., Leloir, L. F., Cardini, C. E., and Paladini, A. C., *J. Biol. Chem.*, **184,** 333–50 (1950)
19. Klein, H. P., *J. Bact.*, **61,** 524–25 (1951)
20. Lampen, J. O., Gest, H., and Sowden, J. C., *J. Bact.*, **61,** 97–98 (1951)
21. Rappoport, D. A., Barker, H. A., and Hassid, W. Z., *Arch. Biochem. Biophys.*, **31,** 326 (1951)
22. Kaushal, R., Jowett, P., and Walker, T. K., *Nature*, **167,** 949 (1951)
23. Lampen, J. O., and Peterjohn, H. R., *J. Bact.*, **62,** 281–92 (1951)
24. Marmur, J., and Schlenk, F., *Arch. Biochem. Biophys.*, **31,** 154–55 (1951)
25. Racker, E., *Nature*, **167,** 408–9 (1951)
26. Watt, D., and Werkman, C. H., *Arch. Biochem. Biophys.*, **31,** 383–90 (1951)
27. Harpur, R. P., Johnson, W. J., and Quastel, J. H., *Arch. Biochem. Biophys.*, **31,** 337–50 (1951)
28. Coolidge, T. B., *J. Infectious Diseases*, **88,** 241–42 (1951)
29. Leloir, L. F., *Arch. Biochem. Biophys.*, **33,** 186–90 (1951)
30. Trucco, E. R., *Arch. Biochem. Biophys.*, **34,** 482–83 (1951)
31. Ohlmeyer, P., *J. Biol. Chem.*, **190,** 21–30 (1951)
32. Hochster, R. M., and Quastel, J. H., *Arch. Biochem. Biophys.*, **31,** 278–84 (1951)
33. Meyerhof, O., and Kaplan, A., *Arch. Biochem. Biophys.*, **33,** 282–98 (1951)
34. Zetterström, R., Ernster, L., and Lindberg, O., *Arch. Biochem. Biophys.*, **31,** 113–26 (1951)
35. Dagley, S., Dawes, E. A., and Morrison, G. A., *J. Bact.*, **61,** 433–41 (1951)
36. Jackins, H. C., and Barker, H. A., *J. Bact.*, **61,** 101–14 (1951)
37. Douglas, H. C., *J. Bact.*, **62,** 517–18 (1951)
38. Cardon, B. P., and Barker, H. A., *Arch. Biochem.*, **12,** 165–80 (1947)
39. Volk, W. A., and Pennington, D., *J. Bact.*, **61,** 469–73 (1951)
40. Racker, E., *J. Biol. Chem.*, **190,** 685–96 (1951)

41. Fales, F. W., *J. Biol. Chem.*, **193,** 113–24 (1951)
42. Krebs, E. G., and Wright, R. R., *J. Biol. Chem.*, **192,** 555–59 (1951)
43. Pasternak, V. Z., Sevag, M. G., and Miller, R. E., *J. Bact.*, **61,** 189–93 (1951)
44. Tsuyuki, H., and MacLeod, R. A., *J. Biol. Chem.*, **190,** 711–19 (1951)
45. Cohen, S. S., *J. Biol. Chem.*, **189,** 617–28 (1951)
46. Cohen, S. S., *Bact. Revs.*, **15,** 131–46 (1951)
47. Scott, D. B. M., and Cohen, S. S., *J. Biol. Chem.*, **188,** 509–30 (1951)
48. Horecker, B. L., and Smyrniotis, P. Z., *J. Biol. Chem.*, **193,** 371–81 (1951)
49. Horecker, B. L., Smyrniotis, P. Z., and Seegmiller, J. E., *J. Biol. Chem.*, **193,** 383–96 (1951)
50. de Ley, J., and Cornut, S., *Nature*, **168,** 515–16 (1951)
51. Kulka, D., Hall, A. N., and Walker, T. K., *Nature*, **167,** 905–6 (1951)
52. Roberts, I. Z., and Wolffe, E. L., *Arch. Biochem. Biophys.*, **33,** 165–66 (1951)
53. Stokes, F. N., and Campbell, J. J. R., *Arch. Biochem.*, **30,** 121–25 (1951)
54. Entner, N., and Stanier, R. Y., *J. Bact.*, **62,** 181–86 (1951)
55. Ajl, S., *Bact. Revs.*, **15,** 211–44 (1951)
56. Umbreit, W. W., Smith, P. H., and Oginsky, E. L., *J. Bact.*, **61,** 595–604 (1951)
57. Stern, J. R., and Ochoa, S., *J. Biol. Chem.*, **191,** 161–72 (1951)
58. Ochoa, S., Stern, J. R., and Schneider, M. C., *J. Biol. Chem.*, **193,** 691–702 (1951)
59. Stern, J. R., Shapiro, B., Stadtman, E. R., and Ochoa, S., *J. Biol. Chem.*, **193,** 703–20 (1951)
60. Korkes, S., del Campillo, A., Gunsalus, I. C., and Ochoa, S., *J. Biol. Chem.*, **193,** 721–35 (1951)
61. Carson, S. F., Mosbach, E. H., and Phares, E. F., *J. Bact.*, **62,** 235–38 (1951)
62. Phares, E. F., *Arch. Biochem. Biophys.*, **33,** 173–78 (1951)
63. Mosbach, E. H., Phares, E. F., and Carson, S. F., *Arch. Biochem. Biophys.*, **33,** 179–85 (1951)
64. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **189,** 123–36 (1951)
65. Kaufman, S., Korkes, S., and del Campillo, A., *J. Biol. Chem.*, **192,** 301–12 (1951)
66. McManus, I. R., *J. Biol. Chem.*, **188,** 729–40 (1951)
67. Kaltenbach, J. P., and Kalnitsky, G., *J. Biol. Chem.*, **192,** 629–39 (1951)
68. Kaltenbach, J. P., and Kalnitsky, G., *J. Biol. Chem.*, **192,** 641–49 (1951)
69. Carson, S. F., Foster, J. W., Jefferson, W. E., Phares, E. F., and Anthony, D. S., *Arch. Biochem. Biophys.*, **33,** 448–58 (1951)
70. Larsen, H., *J. Biol. Chem.*, **193,** 167–73 (1951)
71. Johns, A. T., *Biochem. J.*, **49,** 559–60 (1951)
72. Tsou, C. L., *Biochem. J.*, **49,** 512–20 (1951)
73. Silliker, J. H., and Rittenberg, S. C., *J. Bact.*, **61,** 653–59 (1951)
74. Silliker, J. H., and Rittenberg, S. C., *J. Bact.*, **61,** 661–73 (1951)
75. Kennedy, E. P., and Barker, H. A., *J. Biol. Chem.*, **191,** 419–38 (1951)
76. Wiame, J. M., and Doudoroff, M., *J. Bact.*, **62,** 187–93 (1951)
77. Fraser, D., and Tolbert, B., *J. Bact.*, **62,** 195–97 (1951)
78. Lindstrom, E. S., Lewis, S. M., and Pinsky, M. J., *J. Bact.*, **61,** 481–87 (1951)
79. Sisler, F. D., and ZoBell, C. E., *Science*, **113,** 511–12 (1951)
80. Rosenblum, E. D., and Wilson, P. W., *J. Bact.*, **61,** 475–80 (1951)
81. Zelitch, I., Rosenblum, E. D., Burris, R. H., and Wilson, P. W., *J. Biol. Chem.*, **191,** 295–98 (1951)

82. Zelitch, I., Rosenblum, E. D., Burris, R. H., and Wilson, P. W., *J. Bact.*, **62,** 747–52 (1951)
83. Gale, E. F., *Biochem. J.*, **48,** 286–90 (1951)
84. Gale, E. F., *Biochem. J.*, **48,** 290–97 (1951)
85. Gale, E. F., and Paine, T. F., Jr., *Biochem. J.*, **48,** 298–301 (1951)
86. Gale, E. F., and van Halteren, M. B., *Biochem. J.*, **50,** 34–43 (1951)
87. Windsor, E., *J. Biol. Chem.*, **192,** 607–9 (1951)
88. Mitchell, H. K., and Houlahan, M. B., *J. Biol. Chem.*, **174,** 883–87 (1948)
89. Adelberg, E. A., Bonner, D. M., and Tatum, E. L., *J. Biol. Chem.*, **190,** 837–41 (1951)
90. Tatum, E. L., and Adelberg, E. A., *J. Biol. Chem.*, **190,** 843–52 (1951)
91. Umbarger, H. E., and Adelberg, E. A., *J. Biol. Chem.*, **192,** 883–89 (1951)
92. Fling, M., and Horowitz, N. H., *J. Biol. Chem.*, **190,** 277–85 (1951)
93. Teas, H. J., *J. Biol. Chem.*, **190,** 369–75 (1951)
94. Wright, B. E., *Arch. Biochem. Biophys.*, **31,** 332–33 (1951)
95. Davis, B. D., *J. Biol. Chem.*, **191,** 315–25 (1951)
96. Stanier, R. Y., Hayaishi, O., and Tsuchida, M., *J. Bact.*, **62,** 355–66 (1951)
97. Hayaishi, O., and Stanier, R. Y., *J. Bact.*, **62,** 691–709 (1951)
98. Evans, W. C., Smith, B. S. W., Linstead, R. P., and Elvidge, J. A., *Nature*, **168** 772–75 (1951)
99. Beerstecher, E., Jr., and Edmonds, E. J., *J. Biol. Chem.*, **192,** 497–504 (1951)
100. Kallio, R. E., *J. Biol. Chem.*, **192,** 371–77 (1951)
101. Epps, H. M. R., and Gale, E. F., *Biochem. J.*, **36,** 619–23 (1942)
102. Boyd, W. L., and Lichstein, H. C., *J. Bact.*, **62,** 711–15 (1951)
103. Cammarata, P. S., and Cohen, P. P., *J. Biol. Chem.*, **193,** 45–52 (1951)
104. Cammarata, P. S., and Cohen, P. P., *J. Biol. Chem.*, **193,** 53–62 (1951)
105. Meister, A., Sober, H. A., and Tice, S. V., *J. Biol. Chem.*, **189,** 577–90 (1951)
106. Billen, D., and Lichstein, H. C., *J. Bact.*, **58,** 215–21 (1949)
107. Schou, M., Grossowicz, N., and Waelsch, H., *J. Biol. Chem.*, **192,** 187–96 (1951)
108. Thayer, P. S., and Horowitz, N. H., *J. Biol. Chem.*, **192,** 755–67 (1951)
109. Burton, K., *Biochem. J.*, **50,** 258–68 (1951)
110. Wood, W. A., and Gunsalus, I. C., *J. Biol. Chem.*, **190,** 403–16 (1951)
111. Manson, L. A., and Lampen, J. O., *J. Biol. Chem.*, **193,** 539–47 (1951)
112. Wang, T. P., and Lampen, J. O., *J. Biol. Chem.*, **192,** 339–47 (1951)
113. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **193,** 481–95 (1951)
114. Kaplan, N. O., Colowick, S. P., and Nason, A., *J. Biol. Chem.*, **191,** 473–83 (1951)
115. Friedman, S., and Gots, J. S., *Arch. Biochem. Biophys.*, **32,** 227–29 (1951)
116. Balis, M. E., and Brown, G. B., *J. Biol. Chem.*, **188,** 217–19 (1951)
117. Kerr, S. E., Seraidarian, K., and Brown, G. B., *J. Biol. Chem.*, **182,** 207–16 (1951)
118. Whiteley, H. R., and Douglas, H. C., *J. Bact.*, **61,** 605–16 (1951)
119. Sutton, W. B., Schlenk, F., and Werkman, C. H., *Arch. Biochem. Biophys.*, **32,** 85–88 (1951)
120. Nossal, P. M., *Biochem. J.*, **49,** 407–13 (1951)
121. Black, S., *Arch. Biochem. Biophys.*, **34,** 86–97 (1951)
122. Tomlinson, N., Campbell, J. J. R., and Trussell, P. C., *J. Bact.*, **61,** 17–25 (1951)
123. Chesters, C. G. C., and Rolinson, G. N., *J. Gen. Microbiol.*, **5,** 553–58 (1951)
124. Nason, A., Kaplan, N. O., and Colowick, S. P., *J. Biol. Chem.*, **188,** 397–406 (1951)

125. Delwiche, E. A., *J. Bact.*, **62,** 717–22 (1951)
126. Yanofsky, C., and Bonner, D. M., *J. Biol. Chem.*, **190,** 211–18 (1951)
127. Stadtman, E. R., Novelli, G. D., and Lipmann, F., *J. Biol. Chem.*, **191,** 365–76 (1951)
128. Craig, J. A., and Snell, E. E., *J. Bact.*, **61,** 283–91 (1951)
129. Lichstein, H. C., and Gilfillan, R. F., *Proc. Soc. Exptl. Biol. Med.*, **77,** 459–61 (1951)
130. Lichstein, H. C., *Vitamins & Hormones*, **9,** 27–74 (1951)
131. Davis, B. D., *J. Bact.*, **62,** 221–30 (1951)
132. Ben-Ishai, R., Volcani, B., and Bergmann, E. D., *Arch. Biochem. Biophys.*, **32,** 229–30 (1951)
133. Davis, B. D., *J. Exptl. Med.*, **94,** 243–54 (1951)
134. Oginsky, E. L., Smith, P. H., Tonhazy, N. E., Umbreit, W. W., Lichstein, H. C., and Carson, S. F., *J. Bact.*, **61,** 581–90 (1951)
135. Jukes, T. H., and Stokstad, E. L. R., *Vitamins & Hormones*, **9,** 1–26 (1951)
136. Kearney, E. B., and Englard, S., *J. Biol. Chem.*, **193,** 821–34 (1951)
137. Seeley, H. W., and Rio-Estrada, C. del, *J. Bact.*, **62,** 649–56 (1951)
138. Peters, I. I., and Nelson, F. E., *J. Bact.*, **61,** 591–93 (1951)
139. Shive, W., *Vitamins & Hormones*, **9,** 75–130 (1951)
140. Wright, L. D., *Vitamins & Hormones*, **9,** 131–59 (1951)
141. Lichstein, H. C., and Boyd, R. B., *J. Bact.*, **62,** 415–23 (1951)
142. Reed, L. J., DeBusk, B. G., Gunsalus, I. C., and Hornberger, C. S., Jr., *Science*, **114,** 93–94 (1951)
143. Reed, L. J., DeBusk, B. G., Johnston, P. M., and Getzendaner, M. E., *J. Biol. Chem.*, **192,** 851–58 (1951)
144. Reed, L. J., Getzendaner, M. E., DeBusk, B. G., and Johnston, P. M., *J. Biol. Chem.*, **192,** 859–65 (1951)
145. Lytle, V. L., Zulick, S. M., and O'Kane, D. J., *J. Biol. Chem.*, **189,** 551–55 (1951)
146. Hehre, E. J., *J. Biol. Chem.*, **192,** 161–74 (1951)
147. Militzer, W., Sonderegger, T. B., Tuttle, L. C., and Georgi, C. E., *Arch. Biochem.*, **24,** 75–82 (1949)
148. Militzer, W., Sonderegger, T. B., Tuttle, L. C., and Georgi, C. E., *Arch. Biochem.*, **26,** 299–306 (1950)
149. Militzer, W., Tuttle, L. C., and Georgi, C. E., *Arch. Biochem. Biophys.*, **31,** 416–23 (1951)
150. Stark, E., and Tetrault, P. A., *J. Bact.*, **62,** 247–48 (1951)
151. Georgi, C. E., Militzer, W., Burns, L., and Heotis, J., *Proc. Soc. Exptl. Biol. Med.*, **76,** 598–601 (1951)
152. Mudd, S., Brodie, A. F., Winterscheid, L. C., Hartman, P. E., Beutner, E. H., and McLean, R. A., *J. Bact.*, **62,** 729–39 (1951)
153. Kiessling, W., and Meyerhof, O., *Biochem. Z.*, **296,** 410 (1938)

NUTRITION OF MICROORGANISMS[1]

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This review includes information on the nutrition of bacteria, fungi, algae, and protozoa. Since there are few basic nutritional differences among these microbial groups, or indeed among all living systems, only a limited segregation of the data has been made. Also, the literature on growth factors continues to expand at a very rapid rate and this makes it necessary to introduce some background material into any comprehensible discussion of current investigations. This has been done in the present review but only to a limited extent since much background material is included in the reviews of the past year on the nutrition of bacteria and fungi by Krehl & Llao (1) and of protozoa by Kidder (2). Finally, although a major portion of current investigations is concerned with growth factors and amino acids, as has been true for those of the past decade, yet other aspects of microbial nutrition are important and an attempt has been made to give these other phases adequate treatment.

Carbon and Energy

Convincing evidence is presented by Temple & Colmer (3) that *Thiobacillus ferrooxidans* can grow autotrophically by means of the energy derived from the oxidation of ferrous iron. The oxidation of 120 gm. of $FeSO_4 \cdot 7H_2O$ resulted in the fixation of 16.05 mg. of carbon, an efficiency of 3.2 per cent. The organism is very unusual since it can also grow autotrophically with thiosulfate as the energy source. It is morphologically indistinguishable from *Thiobacillus thiooxidans* and like the latter, it is a strict autotroph being incapable of growing with glucose, peptone, or organic iron compounds. These experiments on *Thiobacillus ferrooxidans* offer the best proof so far available for Winogradsky's contention that there are microorganisms which can grow with the energy derived from the oxidation of ferrous iron.

There is considerable interest in the nutrition of sulfate-reducing bacteria. Senez (4) reports that on an equimolar basis, pyruvate supports approximately twice as much growth of *Sporovibrio desulfuricans* as lactate whereas the latter, because of its greater hydrogen content, leads to the formation of twice as much hydrogen sulfide as pyruvate. Senez therefore rightly emphasizes that in comparing the utilization of different carbon sources by the sulfate-reducing bacteria, a distinction should be made between the effect of the compound on growth and on H_2S formation. As has been noted by other investigators, Senez finds that the addition of 0.1 per cent yeast extract increases the rate and extent of growth of *Sporovibrio*

[1] The survey of the literature pertaining to this review was concluded December 31, 1951.

desulfuricans in both pyruvate and lactate media. Postgate (5) also recommends the use of yeast extract for massive growth of this organism and has shown, more specifically, that cysteine, serine, ornithine, and isoleucine stimulate growth. Although some strains of sulfate-reducing bacteria can grow autotrophically with molecular hydrogen as the sole source of energy, not all strains possess this property [Sisler & Zobell (6)]. Of 39 pure cultures of sulfate-reducing bacteria isolated from marine sediments, only 11 strains grew in an inorganic medium in the presence of hydrogen. However, 33 strains metabolized hydrogen when grown on lactate.

Pseudomonas putrefaciens, which appears to be unable to oxidize or grow with fructose, can do both if relatively high concentrations of fructose are used [Klein (7)]. Thus a concentration above 0.01 *M* fructose must be used to obtain appreciable oxidation. No increase in cells occurs with 1 per cent fructose but a tenfold increase is obtained with 3 per cent fructose. Chemical analyses show that fructose itself is being utilized rather than impurities in the fructose.

Stadtman & Barker (8) have isolated a new methane bacterium, *Methanococcus vannielii*. The organism is a motile coccus that ferments formate to methane, carbon dioxide, and hydrogen and, surprisingly, appears to be unable to use any organic compound other than formate as an energy source.

Ackart & Murray (9) obtained extensive growth of *Mycobacterium avium* in three days at 37°C. in a modified Dubos medium containing succinic acid as the carbon source.

Although it has generally been believed that streptomycin is resistant to microbial decomposition, Pramer & Starkey (10) recently succeeded in isolating from soil and from slime obtained from a streptomycin waste disposal system, a Pseudomonas-like organism which can grow in a mineral salts medium containing streptomycin as the sole organic constituent. Growth of the bacterium is accompanied by inactivation, and therefore undoubtedly decomposition, of the streptomycin.

The growth of *Chlorella vulgaris* in air, both in the light and in the dark, is greatly stimulated by the addition to Emerson's medium of 1 per cent glucose, fructose, and a few other carbohydrates [Neish (11)]. However, most of the carbohydrates tested, and these included pentoses, sugar acids, sugar alcohols, and polysaccharides, were inactive. The amounts of glucose utilized, and cell crop and oxygen formed, were essentially the same irrespective of whether the illuminated glucose cultures were started out in air or in nitrogen. This result is perhaps to be expected since anaerobiosis can only be transitory in cultures which are photosynthesizing and therefore producing oxygen.

Nitrogen

The list of microorganisms which utilize molecular nitrogen has been extended to include all known species of *Rhodopseudomonas*, *i.e. gelatinosa*, *spheroides*, *capsulatus*, and *palustris*, and also *Rhodomicrobium vannielii*

[Lindstrom, Lewis & Pinsky (12)]. These results together with the previously published data on *Rhodospirillum rubrum* by Kamen & Gest (13) and on *Chromatium* and *Chlorobacterium* by Lindstrom, Tove & Wilson (14) suggest that all photosynthetic bacteria can fix molecular nitrogen. In any event, all of the considerable number of strains so far tested do possess this property except possibly one strain of *Rhodopseudomonas*. Some nitrogen-fixing bacteria contain the enzyme, hydrogenase, and the metabolism of hydrogen and nitrogen appear to be intimately related. However, not all organisms possessing hydrogenase can fix N_2. Thus Lindstrom *et al.* (12) could not detect N_2 fixation by *Escherichia coli* and *Hydrogenomonas* although these organisms possess an active hydrogenase system.

Evidence is presented by Sisler & ZoBell (15) that hydrogen-utilizing strains of sulfate-reducing bacteria are able to fix N_2. Lurie (16) suggests the possibility that several species of *Sporotrichum* can fix N_2. The evidence offered is not convincing since growth in a mineral salts, maltose medium free of added nitrogen was slight, in the order of 5 mg. (dry wt.) of cell material per 50 ml. of medium even after four weeks of incubation. Also chemical analysis showed that the increase in nitrogen in such cultures averaged less than 0.1 mg. per 100 ml. of culture. Experiments with N^{15} are needed.

As in the case of bacteria, the list of nitrogen-fixing, blue-green algae continues to lengthen. Fogg (17) isolated *Mastigocladus laminosus* Cohn, a blue-green alga found in hot springs, in pure culture and found that it could grow well in a medium free of added fixed nitrogen. Nitrogen analyses indicate a low but definite fixation of 2.48 mg. of nitrogen per 100 ml. of medium in 20 days. This appears to be the first instance of N_2 fixation by a blue-green alga belonging to a family other than the *Nostocaceae*.

In an attempt to obtain further information concerning possible intermediates in N_2 fixation by *Clostridium pasteurianum*, Rosenblum & Wilson (18) tested hydroxylamine and hydrazine at nontoxic levels and found that they cannot serve as sources of nitrogen for growth. The oximes of pyruvate, oxalacetate, and α-ketoglutarate are utulized but at a low rate. Nitrite and nitrate are suitable as nitrogen sources but only after a lag period. The nitrogen in hyponitrite and nitrous acid appears to be unavailable for growth.

Saccharomyces cerevisiae is able to use either adenine, guanine, cytosine or allantoin as sole source of nitrogen [DiCarlo, Schultz & McManus (19)]. However, *Torula utilis* is much more versatile and can utilize almost all of the naturally occurring purines and pyrimidines. Reischer (20) finds that *Saprolegniaceae* grow well with ammonium salts provided that fumarate, malate, or succinate is in the medium.

Minerals

Calcium is necessary for full growth of *Bacillus megatherium* if the nitrogen in the medium consists of proteins such as serum, albumin, or gelatine [Gorini & Audrain (21)]. The calcium appears to be required for the activation of proteolytic enzymes involved in the decomposition of the proteins

since calcium is not required if the proteins are pre-digested with trypsin or if ammonium chloride is used.

In an extension of his earlier studies, Webb (22) finds that bacteria are less dependent upon magnesium for growth in chemically defined media containing mixtures of amino acids as compared to media with a single source of nitrogen. Gram positive bacilli require more magnesium for growth than gram negative bacteria. Also, a partial deficiency of magnesium inhibits cell division of gram positive rods and causes the formation of filamentous cells.

Shankar & Bard (23) report that *Clostridium perfringens* requires sodium, potassium, calcium, magnesium, and ferrous iron for optimum growth. 2.5 μg of Ca^{++} and 7.5 μg Mg^{++} per ml. of medium are optimal. Mg^{++} can replace Ca^{++} to a large extent. In the absence of added Ca^{++}, microscopic cellular aggregates form. This effect of Ca^{++} deficiency has also been noted by Foster (24) in the case of *Rhodospirillum*. In the absence of Mg^{++} cell division appears to be inhibited since, as in the experiments of Webb (22), filamentous cells are produced.

By further application of a previously developed biological ion purification procedure, MacLeod (25) could show that *Streptococcus faecalis* requires Mg^{++} and Mn^{++} for growth in addition to K^{+} and $PO_4^{=}$ and that Mn^{++} exerts a sparing effect on the requirement for Mg^{++}. Also, some heterofermentative lactic acid bacteria do not grow well even in complex media containing tryptone and yeast extract unless there is added 1 mg. of Mn^{++} and 0.5 gm. sodium citrate per 100 ml. of medium [Evans & Niven (26)]. Likewise, some fastidious gram negative, rod shaped soil bacteria fail to grow in yeast extract or in tryptone-yeast extract medium unless soil extract is added. For some of these bacteria, the soil extract can be replaced by soluble calcium salts [Taylor (27)], but for many others, vitamin B_{12} must be added even though the medium contains adequate amounts of calcium [Lochhead & Thexton (28)]. These investigations on the lactic acid and soil bacteria emphasize that even complex nitrogenous media may be nutritionally inadequate.

Reisher (29) has developed a medium containing ethylenediamine tetracetic acid as metal carrier which supports heavy growth and sporulation of eleven species of *Saprolegniaceae*. It is noteworthy that none of these species can utilize sulfate as a source of sulfur and must be supplied with reduced sulfur compounds such as sulfide or methionine for growth.

A curious effect of magnesium on the growth and cellulolytic activity of four species of fungi was noted by Siu & Sinden (30). Although practically no visible mycelial growth occurs in cellulose media containing little or no magnesium, yet decomposition of the cellulose is as rapid as in media containing sufficient magnesium to permit luxuriant development of mycelium.

Growth Factors

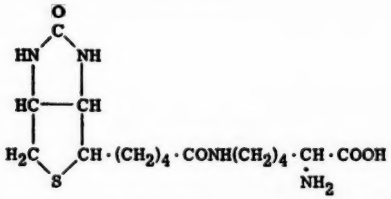
Thiamine.—Reports on the need by fungi of an exogenous supply of thiamine continue to appear. A comprehensive study of the nutrition of the

wheat bunt fungus, *Tilletia caries* discloses a requirement for thiamine or the thiazole portion [Zscheile (31)]. According to Georg (32) strains of *Trichophyton violaceum* grow sparsely but can be maintained on a mineral salts, inorganic nitrogen, glucose, agar medium. However the addition of 1 μg of thiamine per 10 ml. of medium or an equimolar quantity of the pyrimidine moiety greatly enhances growth. Roulet (33) reports that an apparently pure culture of *Phycomyces* requires thiamine or a mixture of its two components for growth. The mold produces mostly oval spores but also an occasional round spore. Strains derived from single round spores require only the pyrimidine component of thiamine. It is not clear why this spore type does not develop when the parent strain is inoculated into media containing only the pyrimidine moiety. Two species of aquatic fungi, *Anguillospora longissima* and *A. gigantea*, grow best in shake cultures at 28°C. and pH 6.5, and reach maximal growth in three to four weeks in a glucose, mineral salts, yeast extract medium [Ranzoni (34)]. The long time required for maximal growth indicates that the medium and growth conditions were far from optimum. For both species, the yeast extract can be replaced by thiamine.

Pyridoxine.—An interesting situation is described by Rabinowitz & Snell (35) in connection with the pyridoxine requirement of *Saccharomyces cerevisiae*. Growth of the yeast occurs in the absence of both thiamine and pyridoxine. But if excess thiamine is added to the medium, pyridoxine becomes essential for growth. This is the reverse of the situation with *Neurospora sitophila*, where thiamine exerts a sparing action on the need for pyridoxine [Stokes, Foster & Woodward (36)]. Cheldelin *et al.* (37) find that pyridoxal phosphate or pyridoxamine phosphate is needed by *Leuconostoc mesenteroides*, strain 10100, for luxuriant growth.

Nicotinic acid.—There is now good evidence for the precursor role of tryptophan in nicotinic acid synthesis in *Neurospora* and also animals. Similar evidence for bacteria is supplied by the experiments of Davis, Henderson & Powell (38) with *Xanthomonas pruni*. For the growth of this bacterium, tryptophan and a number of the postulated niacin precursors, kynurenine, anthranilic acid, 3-hydroxyanthranilic acid, quinolinic acid, and indole are capable of substituting for niacin. Coté & Oleson (39) tested the activity of 22 previously unreported substituted nicotinamide derivatives with *Lactobacillus arabinosus*. Nipecotic acid hydrochloride exhibited about the same growth activity as nicotinic acid, but all the rest were less active or inactive.

Biotin.—An outstanding accomplishment was the determination of the structure and the synthesis of the biotin-complex biocytin by Wright *et al.* (40). This represents the culmination of six years of cooperative work in the Sharp & Dohme and Merck laboratories, and involved the processing of about 5000 lb. of yeast extract with a yield of only a few milligrams of crystalline biocytin. Chemical analysis of the crystalline material indicated that biocytin has the structure of ε-N-biotinyl-L-lysine



This compound was synthesized and found to have chemical and biological properties identical with those of the natural product. Biocytin can be utilized for growth by *Lactobacillus casei*, *L. delbrückii* LD5, *L. acidophilus*, *Streptococcus fecalis* R, *Saccharomyces carlsbergensis* and *Neurospora crassa* but is inactive for *Lactobacillus arabinosus*, *L. pentosus* and *Leuconostoc mesenteroides* P-60. Enzymatic treatment of biocytin containing extracts with pepsin, papain, takadiastase, mylase, or polidase does not cleave the complex and release biotin but acid or alkaline hydrolysis will release biotin.

Andrews & Williams (41), employing *Lactobacillus casei* have confirmed the results of Broquist & Snell (42) which showed that traces of biotin can be detected in microorganisms grown in a medium containing oleic acid as a substitute for biotin. Additional information on the nature of the fatty acids which can substitute for biotin in microbial growth is supplied by Cheng *et al.* (43). They find that for the growth of *Lactobacillus arabinosus*, the *cis*-octadecenoic acids are about as effective as biotin, whereas the *trans* acids, with the exception of elaidic acid, are less active.

d-Biotinol, the alcohol analog of *d*-biotin cannot replace the latter in the nutrition of *Lactobacillus arabinosus*, *L. casei* or *Saccharomyces cerevisiae* [Drekter *et al.* (44)].

Pantothenic acid.—Considerable attention is being devoted to the chemical nature and biological properties of the naturally occurring forms of pantothenic acid, i.e, pantothenic acid conjugate (PAC), coenzyme A and the *Lactobacillus bulgaricus* factor (LBF). Since PAC and coenzyme A appear to contain phosphate, King, Neal & Strong (45) and King & Strong (46) have synthesized two pantothenic acid phosphate derivates, pantolactone phosphate and pantothenic acid diphosphate, and have determined some of their chemical and biological properties. The lactone is inactive for *Saccharomyces cerevisiae* and *Lactobacillus arabinosus* and only very slightly active for *Acetobacter suboxydans*. The pantothenic acid diphosphate resembles PAC and coenzyme A in stability towards acid and alkali and inactivity for *Lactobacillus arabinosus* until treated with intestinal phosphatase. However unlike the latter two compounds, the synthetic diphosphate or its methyl ester is virtually inactive for *Acetobacter suboxydans*. Further investigations along these lines are needed and should be fruitful.

As in the case of PAC and coenzyme A, the microbiological activity of the LBF factor varies with different microorganisms. In general, and in contrast to PAC and coenzyme A, the LBF factor is active for lactic acid bacteria.

But even in this one group of microorganisms wide differences in activity exist [McRorie & Williams (47); Craig & Snell (48)]. Thus LBF is more active than pantothenic acid for *Lactobacillus acidophilus, L. delbrückii, L. bulgaricus* and *L. lactis* but only about one-twelfth as active as pantothenic acid for *Lactobacillus arabinosus* and virtually inactive for *Lactobacillus fermenti.* In comparative tests with 22 microorganisms, synthetic panthethine exhibited the same activity as one of the natural LBF factors [Craig & Snell (48)]. But since Long & Williams (49) have presented evidence for the existence of at least seven LBF factors, further investigations on the nature of the LBF factors are needed.

Acetate-replacing factor, pyruvate oxidase factor and lipoic acid.—The stimulating effect of acetate on the growth of lactic acid bacteria was reported in 1937 by Snell, Tatum & Peterson (50). In 1946, Guirard, Snell & Williams (51), reported the occurrence of an unidentified water-soluble factor in biological preparations which could substitute for acetate in the growth of lactic acid bacteria and which, even in crude concentrates, was more active than acetate. During the past year, Reed *et al.* (52) obtained evidence, by means of chromatographic analyses, of the presence of five acetate-replacing factors in Difco yeast extract. The concentration and some properties of the acetate factors were also described by the same investigators (53). The most active concentrate, obtained from acid hydrolyzed liver or liver residue, supports half-maximum growth of *Streptococcus lactis* at a level of 0.02 mμg. per ml. of medium.

In what initially appeared to be a development unrelated to that of the acetate-replacing factor, O'Kane & Gunsalus (54) discovered that an unidentified factor in yeast extract and other biological materials is essential for the oxidation and dismutation of pyruvate by *Streptococcus faecalis.* It was named the pyruvate oxidase factor (POF). Later, Snell & Broquist (55) showed that POF concentrates could also replace acetate in the growth of lactic acid bacteria. The relationship between POF and the acetate-replacing factor was recently firmly established when through a cooperative effort, Reed *et al.* (56) succeeded in isolating from liver, in crystalline form, a factor which in catalytic amounts can replace the growth promoting activity of acetate for lactic acid bacteria and also activates the oxidative decarboxylation of pyruvate by these bacteria. The active crystalline substance has been named α-lipoic acid. Its exact chemical structure was not reported. As in the case of the *Lactobacillus bulgaricus* multiple factors, additional investigations are necessary to determine the nature of the other acetate-replacing factors present in biological materials.

In the course of a survey of the POF and acetate requirements of a number of lactic acid bacteria, Lytle, Zulick & O'Kane (57) found that several strains, including *Streptococcus faecalis* 734, require either material for growth with gluconate but grow well on glucose in their absence. In gluconate medium, acetate and POF could be replaced with either bicarbonate, malate, or succinate.

Folic acid, vitamin B_{12}, and nucleic acid derivatives.—Tittler & Belsky (58) report that 4-amino-pteroylglutamic acid can substitute for folic acid (pteroylglutamic acid) in the growth of *Tetrahymena geleii*. However, the aminopterin is only about 6 per cent as active as folic acid. Likewise, Dewey, Kidder & Parks (59) find that 4-amino-pteroylglutamic acid and also another folic acid analog, N^{10}-methyl-pteroylgultamic acid, support growth of *T. geleii* in the absence of folic acid. Pteroylaspartic acid and 4-amino-pteroylaspartic acid cannot replace folic acid.

Many soil bacteria will not grow at all or only very poorly in media containing sugars, mineral salts, and yeast extract unless soil extract is added [Lochhead & Chase (60)]. For about 50 per cent of these bacteria, vitamin B_{12} can partially or completely replace the soil extract [Lochhead & Thexton (61)].

According to Soars & Hendlin (62), the loss of activity of vitamin B_{12a} for *Lactobacillus leichmanii* which occurs during autoclaving of the medium can be prevented by the addition of 0.0001 per cent KCN. In the presence of the latter, the unstable vitamin B_{12a} appears be be converted into the stable vitamin B_{12}. The same investigators (63) also report that aniline, xylidene, and *o*-phenylenediamine can partially substitute for vitamin B_{12} in the growth of *Lactobacillus lactis* Dorner. For another lactic acid bacterium, *Lactobacillus bifidus*, the long list of compounds that can be utilized in place of vitamin B_{12} for growth includes desoxyribonucleic acid, thymidine, desoxyadenylic acid, desoxycytidylic acid, desoxyguanylic acid, thymidylic acid, and hypoxanthine desoxyriboside [Skeggs *et al.* (64)].

For the growth of *Torulopsis utilis*, the purine bases are utilized far more readily than the corresponding nucleosides or nucleotides [Kerr, Seraidarian & Brown (65)].

Inositol.—The data of Pennington, Sawyer & Schmidt (66) indicate that the lag in growth of *Saccharomyces cerevisiae* in media deficient in inositol is due to a low rate of synthesis of inositol by constitutive enzymes rather than to the formation of adaptive enzymes. Inositol is also stimulatory and not essential for the growth of *Saccharomyces carlsbergensis* [Smith (67)]. During growth, inositol is rapidly removed from the medium and can be recovered from the yeast by acid hydrolysis. No effect of inositol on the respiration of the yeast could be detected and its metabolic function remains unknown.

Additional information on growth factors is given in the section under the heading, GENERAL.

AMINO ACIDS AND PEPTIDES

A very interesting case of amino acid syntrophism is described by Umbarger & Mueller (68) and Umbarger & Magasanik (69). Two nutritionally deficient mutants, strains 11A16 and 20A19, were isolated with the aid of penicillin from suspensions of *Escherichia coli*, strain K-12, which had been irradiated with ultraviolet light. Strain 11A16 requires isoleucine for

growth and strain 20A19 needs both isoleucine and valine or the corresponding keto acids, α-keto-β-methyl-*n*-valeric acid and α-ketoisovaleric acid. Both of these keto acids were identified in culture filtrates of the isoleucine deficient mutant, 11A16. When the two mutants were inoculated together on a medium deficient in isoleucine and valine both of them grew. This fact could be explained as resulting from the synthesis and excretion of the two keto acids by the isoleucine-requiring mutant which permits the growth of the mutant requiring both isoleucine and valine. The later on growing converts the excess of the keto acid analogue of isoleucine to isoleucine and this in turn permits the growth of the isoleucine mutant.

The accumulation of alanine in cultures of *Brucella abortus* limits the growth of smooth type cells and favors that of nonsmooth mutants. D-Alanine rather than L-alanine is responsible for these effects [Goodlow, Braun & Mika (70)]. Glutamine greatly enhances the utilization of D-glutamic acid for growth of *Lactobacillus arabinosus* [Ayengar, Roberts & Ramasarma (71)]. The inhibitory effect of DL-*o*-hydroxyphenylalanine on the growth of phenylalanine-requiring strains of *Leuconostoc mesenteroides* and *Escherichia coli* is reversed by phenylalanine and to a lesser extent by tryptophan [Volcani, Sicher & Lichtenstein (72)]. Both hydrolyzed casein and glutamic acid inhibit pigment formation by *Serratia*. Synthetic casein-like mixtures of amino acids are inactive which suggests that the inhibitory effect of hydrolyzed casein may be due to substances other than amino acids [Weinberg (73)]. *Corynebacterium diphtheriae*, Toronto strain of Park-Williams no. 8, grows well and produces high titers of toxin in a chemically defined growth factor-amino acid medium [Drew & Mueller (74)]. Ammonium nitrogen is necessary for good growth in addition to amino acids. Holden, Wildman & Snell (75) report that α-hydroxy and α-keto acids corresponding to many of the essential amino acids for *Streptococcus faecalis*, *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* can be utilized by these lactic acid bacteria in place of the corresponding amino acids but only in the presence of vitamin B_6. It appears that utilization of a hydroxy acid proceeds via dehydrogenation to the keto acid, followed by transamination to the amino acid. Only the latter reaction requires vitamin B_6.

For a *Neurospora* mutant which requires either glycine or serine for growth, glyoxylic and glycolic acids are as effective as glycine when the medium is at a pH of 5 to 6 but are essentially inactive at pH 7 [Wright (76)]. Although practically all yeasts so far investigated are capable of growing well in chemically defined media with inorganic nitrogen as sole nitrogen source, there is an occassional yeast which requires amino acids for growth. Two such yeasts are reported by McVeigh & Bell (77). *Candida albicans* Y-475 requires methionine and *Mycoderma vini* Y-939 requires cystine for maximal growth.

Temperature may have a decisive effect on the amino acids required by microorganisms for growth. Thus although an ammonium nitrogen, glucose, mineral salts medium is adequate for the growth of a fecal strain of *Escheri-*

chia coli at 37°C., the addition of glutamic acid and nicotinamide is necessary for growth at 44°C. [Ware (78)]. These results are of importance in connection with the use of the Eijkman test for detection of fecal *E. coli* since absence of growth in the test may be due to nutritional deficiencies in the simple chemically defined media sometimes employed rather than to the source of the coliform bacteria. Likewise, temperature determines the need of *Lactobacillus arabinosus* for certain exogenously required amino acids [Borek & Waelsch (79)]. These investigators have confirmed the earlier results of Lyman *et al.* (80) that at 35°C. or higher, *L. arabinosus* requires phenylalanine, tyrosine, and aspartic acid for growth or full growth and that these amino acids do not have to be supplied if the atmosphere above the cultures is enriched with carbon dioxide. In addition they have shown that when the temperature of incubation is decreased from 39°C. to 26°C., the above three amino acids are no longer needed for growth in air.

Considerable interest has centered about the question of whether peptides of exogenously required amino acids are used directly by microorganisms or undergo hydrolysis prior to utilization. There is some evidence for the utilization of entire peptides. Thus Simmonds & Fruton (81) found that several di- and tri-peptides of proline are considerably more effective than proline in supporting the growth of a prolineless mutant of *E. coli*. More recently Malin, Camien & Dunn (82) showed that glycine, leucine, alanine, and tyrosine dipeptides of glycine are as much as five times more active than glycine for the growth of lactic acid bacteria, especially *Lactobacillus brevis* and *L. pentoaceticus*. Also, *Streptococcus pyogenes*, strain C203, despite the presence of amino acids, requires for full growth a nondialysable peptide preparation obtained from a pancreatic digest of casein [Slade, Knox & Slamp (83)]. And preliminary evidence suggests that the growth factor in serum for pleuropneumonia-like organisms is a protein of low molecular weight or a large polypeptide derivative which is used per se [Smith & Morton (84)].

But in most instances the evidence points to enzymatic hydrolysis of the peptides by the organism prior to use since the peptides are considerably less active than the free essential amino acids contained in them [Simmonds, Tatum & Fruton (85); Simmonds & Fruton (81); Krehl & Fruton (86)]. Thus L-leucine when combined with glycine, tyrosine or proline as di-, tri-, or tetra-peptides increased the lag period of growth of a leucineless mutant of *E. coli* by as much as a day although ultimately the same amount of growth is obtained as with L-leucine [Simmonds, Harris & Fruton (87)]. It seems likely that in these instances, the lag in growth is a measure of the time required by the microorganism to liberate the essential leucine. A relevant observation is that of Dunn & Dittmer (88) who found that di- and tri- peptides of DL-phenylalanine are less active than the free amino acid in reversing the toxicity of β-2-thienyl-DL-alanine for *E. coli* and *Saccharomyces cerevisiae*.

A decision, between direct peptide utilization and preliminary hydrolysis becomes virtually impossible when the peptide permits essentially the same

rate and extent of growth as the corresponding free amino acid. This is the situation encountered by Nurmikko & Virtanen (89) in the case of the utilization of glycine and the glycine peptides, glycylglycine, glycyl-L-leucine, DL-alanylglycine, DL-leucylglycine, and others, by *Leuconostoc mesenteroides* P-60. The above four peptides were also tested by Malin *et al.* (82) with the same organism. The results of the two groups of investigators agree closely in the case of the first two peptides but differ for DL-alanylglycine and DL-leucylglycine which were 73 per cent and 50 per cent respectively as active as glycine in the experiments of Malin *et al.* Virtanen & Nurmikko (90) favor the hypothesis that these glycine peptides are hydrolyzed by the lactic acid bacterium and the free glycine is utilized. Their view is based on the finding that cell suspensions of *L. mesenteroides* when incubated with the peptides, hydrolyze them and liberate free glycine as shown by paper chromatography analysis. Virtanen & Nurmikko are aware, however, that proof of peptide hydrolysis during growth is lacking since in the developing cultures far fewer cells are present than are used in the positive cell suspension experiments. Since their method of analysis was not sensitive enough to follow hydrolysis rates in growing cultures, it is not known whether under growth conditions hydrolysis of the peptides is rapid enough to permit a normal rate of growth of the bacteria.

Effect of Nutrition on Enzyme Formation

The formation of microbial enzymes is profoundly influenced by the availability of metallic ions, growth factors, and amino acids during growth and adaptation. Nason, Kaplan & Colowick (91) find that *Neurospora crassa*, when grown in a zinc deficient medium, contains no alcohol dehydrogenase and a very low concentration of the enzyme which condenses indole and serine to form tryptophan. In contrast, a 10 to 20 fold increase of diphosphopyridine nucleotidase was found in the zinc-deficient mycelial mats, whereas the content of fumarase was unaffected. Biotin deficiency also results in loss of alcohol dehydrogenase and a pronounced increase in diphosphopyridine nucleotidase. Magnesium, manganese, and iron had no effect on the above-mentioned enzymes. Omission of calcium from the medium had an effect similar to that of zinc but not so marked. A deficiency of zinc also causes a marked decrease in protein concentration in the mold. Since these effects of zinc deficiency cannot be overcome by the addition of amino acids, vitamins, purines, and pyrimidines to the medium, it appears that zinc is not involved in the synthesis of these compounds but rather in their subsequent metabolism.

Leuconostoc citrovorum cells, when cultured with growth-limiting amounts of the citrovorum factor, a physiologically active form of folic acid, exhibit only about one-third the respiratory activity of cells cultured with sufficient citrovorum factor for full growth and one-half the capacity to oxidize pyruvic and lactic acids [Chang, Silverman & Keresztesy (92)]. Thiamine is essential for lipase formation by *Candida lipolytica* in a mineral salts, glucose, citrate

medium and maximum lipase activity is obtained with 200 μg. of the vitamin per liter of medium [Peters & Nelson (93)].

The effect of nutrition on the formation by *Escherichia coli* of the three possibly related enzymes, formic hydrogenlyase, formic dehydrogenase, and hydrogenase is reported in several papers. Cells grown in a mineral salts, inorganic nitrogen, glucose medium contain formic dehydrogenase but not formic hydrogenlyase or hydrogenase [Billen & Lichstein (94, 95)]. However, the addition to the medium of a variety of amino acids stimulates the formation of all three enzymes. Likewise, de Ley (96) finds that when the nitrogen content of *E. coli* cells is reduced below 10.7 per cent by growth in a nitrogen deficient, chemically defined medium similar to that of Billen & Lichstein, the formation of formic hydrogenlyase and hydrogenase but not formic dehydrogenase is almost completely suppressed. This nutritional parellelism between formic hydrogenlyase and hydrogenase is additional suggestive evidence of a relationship between the two enzymes [Ordal & Halvorson (97); Waring & Werkman (98)]. A source of energy and amino acids is necessary for the adaptive formation of formic hydrogenlyase by nonproliferating suspensions of *E. coli* [Pinsky & Stokes (99)]. Arginine, aspartic acid, and glutamic acid are essential, four others are stimulatory, and six others inhibitory for formic hydrogenlyase adaptation. The action of the amino acids appears to be direct and specific and suggests that formic hydrogenlyase adaptation involves extensive synthesis of the enzyme from amino acids. The addition of 50 mg. of ammonium nitrate to 100 ml. of a hydrolyzed casein medium completely inhibits the production of the above three enzymes by growing cultures of *E. coli* [Billen (100)]. Nitrate and nitrite also inhibit the adaptive formation of formic hydrogenlyase by nonproliferating cells and the activity of the preformed enzyme [Pinsky & Stokes (99)]. Since formic hydrogenlyase acting independently, or possibly in conjunction with formic dehydrogenase and hydrogenase, is responsible for gas production in cultures of coliform bacteria through decomposition of formate to hydrogen and carbon dioxide, it is understandable why nitrate inhibits gas formation in *E. coli* cultures. This phenomenon was first observed by Pakes & Jollyman in 1901 (101) and is recently emphasized by Tubiash (102) as a possible source of error in the detection of coliform bacteria in water supplies.

Gary & Bard (103) find that nutrition has a marked effect on the carbohydrate metabolism of the Marburg strain of *Bacillus subtilis*. Cells obtained from mineral salts, glucose cultures have little glycolytic activity and oxidize glucose essentially to carbon dioxide and water, whereas cells from a tryptone, yeast extract, glucose medium actively ferment glucose to lactic acid and incompletely oxidize glucose with the formation of acetylmethylcarbinol, acetic acid, carbon dioxide, and water.

Effect of Nutrition on Sporulation

Manganese stimulates the growth and sporulation of several *Bacillus* species [Charney, Fisher & Hegarty (104)]. Growth and sporulation of two

strains of *Bacillus cereus* were obtained in a chemically defined medium consisting of amino acids, yeast nucleic acid, vitamins, and minerals [Williams & Harper (105)]. Valine is the only amino acid essential for growth.

Sporulation in fungi, like that in bacteria, is greatly influenced by nutritional and other environmental factors in ways which at present are far from clear. Relatively abundant growth of the usually sparsely developing fungus, *Microsporum audouini* occurs on an agar medium containing rice infusion, glucose, asparagine, and yeast extract (Hazen (106)]. The evidence on the nature of the nutritional factors required for the formation of macroconidia is inconclusive but sporulation appears to be determined not only by the composition of the medium but also by some inherent and variable property of the fungal strains.

In what appears to be the first instance of a specific stimulation of the sexual cycle of a fungus by a trace element, Basu (107) finds that 10 p.p.m. of calcium stimulates the rate and extent of formation of mature perithecia by *Chaetomium globosum* without affecting mycelial development. Sporulation of this fungus is also stimulated by a contaminating growth of *Aspergillus fumigatus* and active extracts of the latter were obtained [Buston & King (108)].

The complexity of the interrelationships of nutritional and other environmental factors on spore formation in fungi is clearly indicated in a paper by Timnick, Lilly & Barnett (109) on sporulation of *Diaporthe phaseolorum*. Unlike the latter which sporulates best under a combination of light and darkness, *Septoria nodorum* sporulates most abundantly under continuous illumination [Richards (110)]. Of a large number of chemically defined media tested, one composed of galactose, glycine, and mineral salts was most favorable for sporulation. *Sphaerocybe concentrica*, a new hyphomycete, only produces its characteristic coremes on media containing thiamin or a mixture of its two constituents, pyrimidine and thiazole [Magou, Marneffe & Mariat (111)].

General

MacLachlan & Thatcher (112) have investigated the vitamins and amino acids required by *Corynebacterium sepedonicum* for rapid growth. The authors reject the use of direct measurement of growth as an index of the effect of an added nutrient because "Vitamins and amino acids are required in minute quantities by bacteria, and the increase in growth caused by these materials is difficult to detect by cultural methods." This statement is hardly in accord with the fact that an enormous number of nutritional investigations of microorganisms have been successfully carried out by measuring changes in the turbidity, acidity, or cell weight of the cultures. The method adopted by MacLachlan & Thatcher, namely, measurement of oxygen consumption in the Warburg apparatus, cannot be considered an improvement over cultural methods since it is well known that oxygen is consumed by metabolic processes other than those involving growth. Thus,

the authors find that the addition of calcium pantothenate increases oxygen consumption by *Corynebacterium sepedonicum* and therefore presumably growth. Yet our calculations of the amounts of oxgyen consumed per hour indicate a constant rate which is characteristic of nonproliferating cells rather than an increasing rate which one should expect in growing cultures.

Of 19 cultures of *Streptococcus uberis* tested, all require nictotinic acid, pyridoxine, thiamine, riboflavin, pantothenic acid, and biotin, whereas 15 strains also require folic acid. Essential amino acids include tryptophan, phenylalanine, arginine, valine, leucine, and glutamic acid [Seeley (113)]. The growth factor and amino acid requirements of *Leuconostoc dextranicum*, strain "elai," were determined and in no instance was a nutrient found to be more essential for dextran synthesis than for growth in sucrose media [Whiteside-Carlson & Rosano (114)]. In sharp contrast to the large assortment of vitamins and amino acids usually needed by lactobacilli for growth, *Lactobacillus bifidus* needs only a single amino acid, cysteine and two vitamins, biotin and pantothenic acid [Hassinen *et al.* (115)].

Of 57 cultures of nonpathogenic *Neisseria* tested, 47 require either nicotinic acid or the latter and biotin for growth in a glucose, casein hydrolysate medium, and the remaining 10 strains do not need any exogenous vitamins [Nemes, Pelczar & Doetsch (116)]. Pearce & Powell (117) have developed a highly selective medium for the isolation of *Bacillus anthracis* from soils. Addition of 40 μg. of hematin plus 60 μg. of lysozyme per ml. of an agar-peptone medium and incubation at 37.5°C. to 40°C. permits good growth of *B. anthracis* while suppressing more than 95 per cent of the other *Bacillus* species found in soil. Gottlieb & Diamond (118) have devised a chemically defined medium consisting of glycerol, serine, sodium lactate, and mineral salts which supports growth and yields of chloromycetin by *Streptomyces venezuelae* equal to those obtainable in complex nitrogenous media.

Schizosaccharomyes octosporus requires inositol, pantothenic acid, nicotinic acid, biotin, histidine, methionine, and adenine for growth in a glucose, mineral salts, asparagine, ammonium sulfate medium [Northam & Norris (119)]. The need for the addition of plasma to peptone media in order to obtain growth of the yeast phase of *Histoplasma capsulatum* is due to the ability of the plasma to absorb unknown toxic substances from the medium (McVickar (120)]. The insect parasitizing fungi of the genus *Entomophthora* namely, *E. apiculata* and *E. coronata*, have been successfully cultured for the first time in a chemically defined medium consisting of glucose, asparagine, and mineral salts; vitamins or other accessory growth factors are not required [Wolf (121)]. Raper (122) in an excellent paper describes conditions for the isolation, cultivation, and conservation of that fascinating group of microorganisms, the simple slime molds. Cantino (123) reports that an unidentified growth factor which is produced by autoclaving fructose with yeast extract is necessary for rapid and vigorous growth of the aquatic fungus, *Pythiogeton*, in media containing fructose as the carbon source. The factor is not required in glucose media.

Trypanosoma cruzi can be continuously propagated in a complex but almost completely defined medium consisting of glucose, amino acids, known growth factors, mineral salts, and heat coagulated red blood cells [Little & Oleson (124)]. Additional data on the effect of organic and inorganic nutrients on the growth of *Tetrahymena geleii* are presented by Huddleston (125). Analysis of the results is complicated by the fact that the effects of the added amino acids and vitamins were determined in a basal medium which contained 1 per cent proteose-peptone, a rich source of amino acids and vitamins.

Much valuable information on the nutrition of algae is presented in the recently published *Manual of Phycology* (126), especially in the chapters on the physiology and biochemistry of the algae by Blinks and on methods for the cultivation of algae by Pringsheim.

Information on the nutritional stability of microorganisms used in the assay of vitamins and amino acids is of obvious importance. In most instances assay microorganisms retain their nutritional characteristics unchanged for years if the medium, temperature of incubation, and other environmental factors are kept constant. However, it is sometimes possible to train or adapt an assay organism to become independent of a required vitamin or amino acid, or to isolate variants or mutants with the latter property. By means of serial transfer in a tryptophan deficient medium for only four days, Sbarra & Hardin (127) were able to train *Lactobacillus arabinosus* 17–5 and *Lactobacillus casei* to grow without tryptophan. The result with *L. arabinosus* is in agreement with the earlier experiments of Wright & Skeggs (128). It was not possible, however, to obtain strains of either species which could dispense with pantothenic acid, nicotinic acid, riboflavin, or pyridoxine. Also, Cheldelin & Nygaard (129) obtained a variant of *Lactobacillus gayoni* 8289 which requires nucleotides, folic acid, and an unknown factor in liver for rapid and luxuriant growth. This variant was changed by frequent transfer in a medium containing suboptimal amounts of nucleotides into one that grows well in the absence of any of the above growth factors.

Conclusions

The discovery of new growth factors for microorganisms has become a relatively rare event. This is not surprising in view of the passage of more than a decade of intensive research in this field. Theoretically, any essential organic component of a microbial cell may be required preformed by some organism which is unable to synthesize it. And the number of such compounds is obviously very large. Yet, in recent years, extensive nutritional surveys of microorganisms disclose, in almost all instances, requirements for known growth factors. This repetition of nutritional patterns emphasizes not only the unity in biological systems but also the anabolic or synthetic self-sufficiency of microorganisms. For despite the fact that at first glance most lactic acid bacteria appear to be nutritionally very dependent on a sup-

ply of preformed materials since they require an exogenous supply of twenty-five to thirty growth factors and amino acids, yet this number of required compounds is small compared to the number which these bacteria synthesize in the normal course of their development.

This biological limitation to the discovery of new growth factors has undoubtedly played a part in establishing the present trend towards more intensive investigations of the known growth factors. These investigations have led to the acquisition of much valuable information on the naturally occurring forms of pantothenic acid, pyridoxine, folic acid, biotin, and other growth factors. And the available evidence suggests that such forms are generally intermediates between the usual, commercially available vitamins and the metabolically functional units, the coenzymes. These investigations should be of the utmost importance in elucidating the chemical nature of the many still unidentified coenzymes. Most investigations of this type have been carried out with bacteria and yeasts, but similar work needs to be done with the fungi and other microorganisms.

But there are other phases of nutrition which are important and which in many instances have so far not received the systematic investigation which they merit. Thus there are the subtler aspects of nutrition in which a nutrient may have little or no effect on the extent of growth but may greatly stimulate the rate of growth, or, more importantly, exert a profound effect on metabolic processes such as the formation of specific enzymes, spores, capsular material, pigments, toxins, and others. Also, environmental factors such as temperature, pH, carbon dioxide concentration, and others greatly influence nutritional requirements. Both in laboratory cultures and in nature, the microbial environment constantly changes. More information how such changes influence nutrition is necessary for a better understanding not only of microbial nutrition but also of microbial ecology.

LITERATURE CITED

1. Krehl, W. A., and Llao, S. J., *Ann. Rev. Microbiol.*, **5**, 121–38 (1951)
2. Kidder, G. W., *Ann. Rev. Microbiol.*, **5**, 139–56 (1951)
3. Temple, K. L., and Colmer, A. R., *J. Bact.*, **62**, 605–11 (1951)
4. Senez, J., *Ann. inst. Pasteur*, **80**, 395–408 (1951)
5. Postgate, J. R., *J. Gen. Microbiol.*, **5**, 714–24 (1951)
6. Sisler, F. D., and Zobell, C. E., *J. Bact.*, **62**, 117–27 (1951)
7. Klein, H. P., *J. Bact.*, **61**, 524–25 (1951)
8. Stadtman, T. C., and Barker, H. A., *J. Bact.*, **62**, 269–80 (1951)
9. Ackart, W. B., and Murray, T. J., *J. Bact.*, **62**, 75–79 (1951)
10. Pramer, D., and Starkey, R. L., *Science*, **113**, 127 (1951)
11. Neish, A. C., *Can. J. Botany*, **29**, 68–78 (1951)
12. Lindstrom, E. S., Lewis, S. M., and Pinsky, M. J., *J. Bact.*, **61**, 481–87 (1951)
13. Kamen, M. D., and Gest, H., *Science*, **109**, 560 (1949)
14. Lindstrom, E. S., Tove, S. R., and Wilson, P. W., *Science*, **112**, 197–98 (1950)
15. Sisler, F. D., and ZoBell, C. E., *Science*, **113**, 511–12 (1951)
16. Lurie, H. I., *Mycologia*, **43**, 117–29 (1951)
17. Fogg, G. E., *J. Exptl. Botany*, **2**, 117–20 (1951)
18. Rosenblum, E. D., and Wilson, P. W., *J. Bact.*, **61**, 475–80 (1951)
19. DiCarlo, F. J., Schultz, A. S., and McManus, D. K., *J. Biol. Chem.*, **189**, 151–57 (1951)
20. Reischer, H. E., *Mycologia*, **43**, 319–28 (1951)
21. Gorini, L., and Audrain, L., *Biochim. et Biophys. Acta*, **6**, 477–86 (1951)
22. Webb, M., *J. Gen. Microbiol.*, **5**, 485–95 (1951)
23. Shankar, K., and Bard, R. C., *J. Bact.*, **63**, 279–90 (1952)
24. Foster, J. W., *J. Bact.*, **47**, 355–72 (1947)
25. MacLeod, R. A., *J. Bact.*, **62**, 337–45 (1951)
26. Evans, J. B., and Niven, C. F., Jr., *J. Bact.*, **62**, 599–603 (1951)
27. Taylor, C. B., *Nature*, **168**, 115 (1951)
28. Lochhead, A. G., and Thexton, R. H., *Nature*, **167**, 1034 (1951)
29. Reischer, H. S., *Mycologia*, **43**, 142–55 (1951)
30. Siu, R. G. H., and Sinden, J. W., *Am. J. Botany*, **38**, 284–90 (1951)
31. Zscheile, F. P., *Phytopathology*, **41**, 1115–24 (1951)
32. Georg, L. K., *Mycologia*, **43**, 297–309 (1951)
33. Roulet, M. A., *Experientia*, **7**, 177–78 (1951)
34. Ranzoni, F. V., *Mycologia*, **43**, 130–41 (1951)
35. Rabinowitz, J. C., and Snell, E. E., *Arch. Biochem. Biophys.*, **33**, 472–81 (1951)
36. Stokes, J. L., Foster, J. W., and Woodward, C. R., Jr., *Arch. Biochem.*, **2**, 235–45 (1943)
37. Cheldelin, V. H., Nygaard, A. P., Kornberg, H. A., and Williams, R. J., *J. Bact.*, **62**, 134–35 (1951)
38. Davis, D., Henderson, L. M., and Powell, D., *J. Biol. Chem.*, **189**, 543–49 (1951)
39. Coté, L., and Oleson, J. J., *J. Bact.*, **61**, 463–67 (1951)
40. Wright, L. D., Cresson, E. L., Skeggs, H. R., Peck, R. L., Wolf, D. E., Wood, T. R., Valiant, J., and Folkers, K., *Science*, **114**, 635–36 (1951)
41. Andrews, E. A., and Williams, V. R., *J. Biol. Chem.*, **193**, 11–12 (1951)
42. Broquist, H. P., and Snell, E. E., *J. Biol. Chem.*, **188**, 431–44 (1951)

43. Cheng, A. L. S., Greenberg, S. M., Deuel, H. J., Jr., and Melnick, D., *J. Biol. Chem.*, **192,** 611–22 (1951)
44. Drekter, L., Scheiner, J., deRitter, E., and Rubin, S. H., *Proc. Soc. Exptl. Biol. Med.*, **78,** 381–83 (1951)
45. King, T. E., Neal, A. L., and Strong, F. M., *J. Biol. Chem.*, **189,** 307–14 (1951)
46. King, T. E., and Strong, F. M., *J. Biol. Chem.*, **189,** 315–23 (1951)
47. McRorie, R. A., and Williams, W. L., *J. Bact.*, **61,** 737–45 (1951)
48. Craig, J. A., and Snell, E. E., *J. Bact.*, **61,** 283–91 (1951)
49. Long, C. L., and Williams, W. L., *J. Bact.*, **61,** 195–202 (1951)
50. Snell, E. E., Tatum, E. L., and Peterson, W. H., *J. Bact.*, **33,** 207–25 (1937)
51. Guirard, B. M., Snell, E. E., and Williams, R. J., *Arch. Biochem.*, **9,** 381 (1946)
52. Reed, L. J., DeBusk, B. G., Johnston, P. M., and Getzendaner, M. E., *J. Biol. Chem.*, **192,** 851–58 (1951)
53. Reed, L. J., Getzendaner, M. E., DeBusk, B. G., and Johnston, P. M., *J. Biol. Chem.* **192,** 859–65 (1951)
54. O'Kane, D. J., and Gunsalus, I. C., *J. Bact.*, **54,** 20–21 (1947)
55. Snell, E. E., and Broquist, H. P., *Arch. Biochem.*, **23,** 326–38 (1949)
56. Reed, L. J., DeBusk, B. G., Gunsalus, I. C., and Hornberger, C. S., Jr., *Science*, **114,** 93–94 (1951)
57. Lytle, V. L., Zulick, S. M., and O'Kane, D. J., *J. Biol. Chem.*, **189,** 551–55 (1951)
58. Tittler, I. A., and Belsky, M. M., *Science*, **114,** 493–94 (1951)
59. Dewey, V. C., Kidder, G. W., and Parks, R. E., *Proc. Soc. Exptl. Biol. Med.*, **78,** 88–91 (1951)
60. Lochhead, A. G., and Chase, F. E., *Soil Sci.*, **55,** 185–95 (1943)
61. Lochhead, A. G., and Thexton, R. H., *Nature*, **167,** 1034 (1951)
62. Soars, M. H., and Hendlin, D., *J. Bact.*, **62,** 15–17 (1951)
63. Hendlin, D., and Soars, M. H., *J. Bact.*, **62,** 633–38 (1951)
64. Skeggs, H. R., Nepple, H. M., Spizizen, J., and Wright, L. D., *J. Bact.*, **61,** 41–45 (1951)
65. Kerr, S. E., Seraidarian, K., and Brown, G. B., *J. Biol. Chem.*, **188,** 207–16 (1951)
66. Pennington, D., Sawyer, C. H., and Schmidt, J., *J. Bact.*, **62,** 677–83 (1951)
67. Smith, R. H., *J. Gen. Microbiol.*, **5,** 772–80 (1951)
68. Umbarger, H. E., and Mueller, H. J., *J. Biol. Chem.*, **189,** 277–85 (1951)
69. Umbarger, H. E., and Magasanik, B., *J. Biol. Chem.*, **189,** 287–92 (1951)
70. Goodlow, R. J., Braun, W., and Mika, L. A., *Arch. Biochem.*, **30,** 402–6 (1951)
71. Ayengar, P., Roberts, E., Ramasarma, G. B., *J. Biol. Chem.*, **193,** 781–91 (1951)
72. Volcani, B. E., Sicher, S., and Lichtenstein, S., *J. Biol. Chem.*, **192,** 543–49 (1951)
73. Weinberg, E. D., *J. Bact.*, **62,** 785–92 (1951)
74. Drew, R. M., and Mueller, J. H., *J. Bact.*, **62,** 549–59 (1951)
75. Holden, J. T., Wildman, R. B., and Snell, E. E., *J. Biol. Chem.*, **191,** 559–76 (1951)
76. Wright, B. E., *Arch. Biochem. Biophys.*, **31,** 332–33 (1951)
77. McVeigh, I., and Bell, E., *Bull. Torrey Botan. Club*, **78,** 134–43 (1951)
78. Ware, G. C., *J. Gen. Microbiol.*, **5,** 880–84 (1951)
79. Borek, E., and Waelsch, H., *J. Biol. Chem.*, **190,** 191–96 (1951)
80. Lyman, C. M., Moseley, O., Wood, S., Butler, B., and Hale, F., *J. Biol. Chem.*, **167,** 177–87 (1947)

81. Simmonds, S., and Fruton, J. S., *J. Biol. Chem.*, **174,** 705–15 (1948)
82. Malin, R. B., Camien, M. N., and Dunn, M. S., *Arch. Biochem. Biophys.*, **32,** 106–12 (1951)
83. Slade, H. D., Knox, G. A., and Slamp, W. C., *J. Bact.*, **62,** 669–75 (1951)
84. Smith, P. F., and Morton, H. E., *J. Bact.*, **61,** 395-405 (1951)
85. Simmonds, S., Tatum, E. L., and Fruton, J. S., *J. Biol. Chem.*, **169,** 91–101 (1951)
86. Krehl, W. A., and Fruton, J. S., *J. Biol. Chem.*, **173,** 479–85 (1948)
87. Simmonds, S., Harris, J. I., and Fruton, J. S., *J. Biol. Chem.*, **188,** 251–62 (1951)
88. Dunn, F. W., and Dittmer, K. D., *J. Biol. Chem.*, **188,** 263–72 (1951)
89. Nurmikko, V., and Virtanen, A. I., *Acta Chem. Scand.*, **5,** 97-101 (1951)
90. Virtanen, A. I., and Nurmikko, V., *Acta Chem. Scand.*, **5,** 681-89 (1951)
91. Nason, A., Kaplan, N. O., and Colowick, S. P., *J. Biol. Chem.*, **188,** 397–406 (1951)
92. Chang, S. C., Silverman, M., and Keresztesy, J. C., *J. Bact.*, **62,** 753–62 (1951)
93. Peters, I. I., and Nelson, F. E., *J. Bact.*, **61,** 591–93 (1951)
94. Billen, D., and Lichstein, H. C., *J. Bact.*, **60,** 311–14 (1950)
95. Billen, D., and Lichstein, H. C., *J. Bact.*, **61,** 515–22 (1951)
96. de Ley, J., *Bull. soc. chim. biol.*, **33,** 505–7 (1951)
97. Ordal, E., and Halvorson, H. O., *J. Bact.*, **38,** 199–220 (1939)
98. Waring, W. S., and Werkman, C. H., *Arch. Biochem.*, **4,** 75–87 (1944)
99. Pinsky, M. J., and Stokes, J. L., *J. Bact.* (In press)
100. Billen, D., *J. Bact.*, **62,** 793–97 (1951)
101. Pakes, W. C. C., and Jollyman, W. H., *J. Chem. Soc.*, **79,** 459–61 (1901)
102. Tubiash, H. S., *Am. J. Pub. Health*, **41,** 833–38 (1951)
103. Gary, N., and Bard, R. C. (Unpublished data)
104. Charney, J., Fisher, W. P., and Hegarty, C. P., *J. Bact.*, **62,** 145–48 (1951)
105. Williams, O. B., and Harper, O. F., Jr., *J. Bact.*, **61,** 551–56 (1951)
106. Hazen, E. L., *Mycologia*, **43,** 284–96 (1951)
107. Basu, S. N., *J. Gen. Microbiol.*, **5,** 231–38 (1951)
108. Buston, H. W., and King, E. J., *J. Gen. Microbiol.*, **5,** 766–71 (1951)
109. Timnick, M. B., Lilly, V. G., and Barnett, H. L., *Phytopathology*, **41,** 327–36 (1951)
110. Richards, G. S., *Phytopathology*, **41,** 571–78 (1951)
111. Magrou, J., Marneffe, H., and Mariat, F., *Ann. inst. Pasteur*, **80,** 143–50 (1951)
112. MacLachlan, D. S., and Thatcher, F. S., *Can. J. Botany*, **29,** 246–59 (1951)
113. Seeley, H. W., *J. Bact.*, **62,** 107–15 (1951)
114. Whiteside-Carlson, V., and Rosano, C. L., *J. Bact.*, **62,** 583–89 (1951)
115. Hassinen, J. B., Durbin, G. I., Tomarelli, R. M., and Bernhart, F. W., *J. Bact.*, **62,** 771–77 (1951)
116. Nemes, J. L., Pelczar, M. J., and Doetsch, R. N., *J. Infectious Diseases*, **88,** 156–57 (1951)
117. Pearce, T. W., and Powell, E. O., *J. Gen. Microbiol.*, **5,** 387–90 (1951)
118. Gottlieb, D., and Diamond, L., *Bull. Torrey Botan. Club*, **78,** 56–60 (1951)
119. Northam, B. E., and Norris, F. W., *J. Gen. Microbiol.*, **5,** 502–7 (1951)
120. McVickar, D. L., *J. Bact.*, **62,** 137–43 (1951)
121. Wolf, F. T., *Bull. Torrey Botan. Club*, **78,** 211–20 (1951)
122. Raper, K. B., *Quart. Rev. Biol.*, **26,** 169–90 (1951)
123. Cantino, E. C., *Am. J. Botany*, **38,** 579–85 (1951)

124. Little, P. A., and Oleson, J. J., *J. Bact.*, **61,** 709–14 (1951)
125. Huddleston, M. S., *Physiol. Zool.*, **24,** 141–55 (1951)
126. *Manual of Phycology* (Smith, G. M., Ed., Chronica Botanica Co., Waltham, Mass., 375 pp., 1951)
127. Sbarra, A. J., and Hardin, M. M., *J. Bact.*, **61,** 99–100 (1951)
128. Wright, L. D., and Skeggs, H. R., *J. Biol. Chem.*, **159,** 611–16 (1945)
129. Cheldelin, V. H., and Nygaard, A. P., *J. Bact.*, **61,** 489–95 (1951)

GENETICS OF MICROORGANISMS[1]

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The introduction of bacteria, fungi, algae, and protozoa to genetics has stimulated this science to new developments. The value of microorganisms for genetical research has many reasons. Rapid multiplication permits examination of many cell generations in a short time. The smallness of individuals allows small culture spaces, constant environmental conditions, and treatment of large numbers of individuals. Simple selection methods for interesting phenotypes are possible and permit a broad investigation of rare processes like mutation or close linkage. Because many of the organisms are unicellular, isolation of almost every cell for genetical analysis is possible. All these advantages are so desirable that it is no wonder that the first steps to use microorganisms, especially bacteria, for genetical work had been taken independently in different countries. Whereas the genetics of microorganisms with a "real" nucleus, fungi, algae, and protozoa, could be related rather quickly to the genetics of higher organisms, the seemingly "anuclear" bacteria had to be studied extensively to determine whether they make use of conventional genetical mechanisms. This examination, which can now be considered finished, has led to the conclusion that the heriditary apparatus of bacteria does not differ essentially from that of higher organisms. But some phenomena studied further in the last years in bacteria, especially the action of infectious transformation substances or cytoplasmic inheritance, may guide us perhaps to some information about the "genetical substance" which could not be revealed by classical genetics.

The task of this paper is to review the progress in microbial genetics in 1950 and 1951, including some earlier investigations of the author which are scarcely known outside Germany. Purely biochemical analysis by the aid of mutants [Catcheside (47); Horowitz (124); Horowitz & Mitchell (125); Ryan (245)] and the genetics of phages and viruses are excluded.

A survey of the whole field is available now in a book by Catcheside (49). The genetics of bacteria has been reviewed by Boivin (31), Cavalli-Sforza (53), Gottschewski (116), and Kaplan (138, 143). Collections of general methods are given for *Neurospora* [Ryan (245)], yeast [Roberts (230)], *Paramecium* [Sonneborn (262)], biochemical mutants [Lederberg (159)], and drug-resistant mutants [Witkin (308)].

A number of progressive methods for special work have been developed. These include an apparatus ("chemostat") for continuous growth of bacterial cultures [Monod (192); Novick & Szilard (208, 209)]; a device for production of pure, smooth cell cultures [Braun & Howell (37)]; a plate for a

[1] The survey of the literature pertaining to this review was concluded in November, 1951.

gradient drug concentration [Szybalski (282)]; a single cell pedigree technique for bacteria [Zelle (319)]; a rapid method of determining the antigenic type of colonies [Stocker (272)]; printing techniques for plate inoculation [Lederberg (161); Visconti di Modrone (295)]; a simple lyophilisation method [Barrat & Tatum (24)]; production of diploid heterozygotes in *Aspergillus* [Roper (238)]; and a structure-saving method for fixing and staining bacterial chromosomes [DeLamater (66, 68)].

Analysis of Mutation Process

Mutation is the elementary act of change in genetical units without recombination by sexual processes; therefore, its analysis promises some insight into the interior of these basic structures. One of its most remarkable traits is the "statistical" occurrence. This character had permitted a first step in the direction of causal understanding of this primary process of development of life. Two principal possibilities to interpret these statistics exist: The variability of cells is caused (*a*) by variable sensitivity of individuals alone to mutating conditions, or (*b*) by the statistical action of mutation-inducing agents. If the second interpretation should be correct, at least partly, it should be possible to deduce from the statistics something about the primary step of mutation. In many cases the probability of mutations, μ, increases according to the function $1\text{-}e^{\mu D}$, and therefore is nearly proportional to the dose D of the inducing agent. The frequency function is exponential. Such a relation is not to be expected for individual sensitivities. In some instances, two different mutation types of the same cells follow two different dose-functions. This is difficult to explain in terms of individual variability, but easy to explain by the hypothesis of statistical effectivity of the agent. The mutagenic agent acts like shot from a shotgun; the mutations behave as though initiated by one or a few hits. It must be mentioned because of some misunderstanding that this "hit or target" theory does not mean that the mutation is caused directly by a hit of a photon, particle, or any energy quantum, but only that the initiating step is the uptake of an effective unit of the agent in a sensitive zone of the cell. This step may or may not be followed by further reactions inside or outside the site of the primary reaction. The indices of "indirect" action of radiations and other agents do not disprove the hit theory. If one wished to disprove it, one must show that the whole mutation statistics are caused by the strange individual variability mentioned above. Thus the theory of Timofeef-Ressovsky, Zimmer & Delbrück (288), that a mutation is an event similar to the reaction of a single molecule as governed by quantum statistics, has been fruitful until now. The assumption that the linear dose proportionality may also be caused by a high number of hits [Opatowski & Christiansen (210)] is an error [Boag (30); Kaplan (142)]. The biophysical analysis of the mutation process by the aid of the hit theory has two aspects: (*a*) elucidation of the initiating reaction, the nature of the hit; (*b*) exploration of the reaction chain or system following this first step and ending in the completed mutation.

A series of new methods for use in mutation analysis has been described recently. Auxotrophic mutations can be concentrated by starvation [Macdonald & Pontecorvo (175, 176); Pontecorvo (216)], and certain rare ones by exclusion of inhibitors [Fries (104)]. Atwood (17, 18) invented a method of finding induced lethal mutations and chromosomal deficiencies in conidia of *Neurospora*. Methods for mutation work in *Penicillium* are described by Arima *et al.* (6, 9, 10). The lag of mutation appearance in *Escherichia coli* could be excluded by sodium nucleate [Witkin (309)]. The frequent colour mutations of a strain of *Serratia marcescens* (*Bacterium prodigiosum*) are useful for mutation work because no special method for mutant selection is necessary [Kaplan (131 to 146)].

Spontaneous mutation.—The classical method of Luria & Delbrück (170a) for determining the pure mutation rate excluding selection consists in making a series of cultures and seeking for the frequency p of cultures without any mutant. The mutation rate per time unit is $\mu = 2.3$ (log p)tN, where t = culture time and N = number of cells per culture. This has been used by Rubin (241) in measuring the mutation rate to streptomycin resistance, and by Bertani (26) in measuring the rate of prototrophic mutations in *E. coli* B/Sd 4. A value of 1.4×10^{-8} per fission was obtained.

A small prototrophy rate of 6.7×10^{-9} was found in *Aspergillus nidulans*, strain ly^- (236). In some cases, the mutation rates in both directions, forward and back, could be determined. In *E. coli* 15, the rate h^- to h^+ is 2.8×10^{-8} per generation, the reverse rate being about 50 times higher [Atwood *et al.* (15); Lieb (164, 165)]. The changes of flagellar antigen from group to specific phase in *Salmonella typhimurium* in different strains have the rates, 1×10^{-4} to 5×10^{-3}, the reverse 1×10^{-5} to 8×10^{-4} [Stocker (272)]. Colour mutations of *Serratia marcescens* from red to white appear 0.002 to 0.055 per day, white to red 0.004 to 0.15 in different lines [Kaplan (131, 133)]. In the auxotrophic two-step mutant of *E. coli*, $B/r/h^-/s^-$, the reversion to full prototrophy is as frequent as the reversion to only one requirement. This unusual behaviour is due to suppressor mutation [Demerec *et al.* (74)].

If a mutation has a small probability, a certain number of cells must be present to realise the mutation. This number may be reached by "invisible" growth, as in the case of the itaconate mutation of *Pseudomonas fluorescens* [Englesberg & Stanier (87)]. But cell fission seems unnecessary for the mechanism of mutation. Spontaneous mutation goes on in growing as well as in resting cells of *Serratia* [Kaplan (131)].

The rate of spontaneous mutation is influenced by the genotype: *E. coli* B/r, B/1, and B/6 have different frequencies of mutation to streptomycin resistance [Demerec *et al.* (72)]. In some cases the mutation rate may be influenced in the same manner as growth by the environment, e.g., in *S. typhimurium* by medium and temperature (272). Then it is possible to use the fission period as the time unit. But this is not true in general. Novick & Szilard (209) have reported the rate of T5-resistance mutations as 1.25×10^{-8}

per hr. and to be independent of growth rate variations in the chemostat. In *Serratia* the mutant percentage in colonies of different size but of the same age is equal [Kaplan (131)].

These facts indicate that mutation is not a mistake in gene reproduction, but an event similar to a reaction of a single molecule (131, 132, 209). This hypothesis is supported by the fact that the temperature dependence of colour mutations of *Serratia* corresponds to an activation energy U of about 0.6 ev, using the formula $\mu = Ce^{-U/RT}$ (131, 132). The T5-resistance mutation yields a value of $U \approx 0.45$ ev (209). The energies are in the range of chemical reactions. The frequency factors C, including the entropy changes of the mutating reactions, for colour mutations are in the range of 10^3 per sec., for T5-resistants 10^{-3} per sec., and thus much smaller than for normal reactions, 10^5 to 10^{13} per sec. The same fact had been noted earlier by Timofeef, Zimmer & Delbrück (288) for *Drosophila*. This may indicate that mutation is a sort of transformation decreasing only little, or even increasing, the thermodynamic probability, i.e., preserving or raising the complexity of the reacting gene "molecule." The finding that different mutation steps have different C values means, in terms of this hypothesis, that the reacting atom-groups are not the same for different steps (280). It is unlikely that spontaneous mutation is initiated by thermal vibration alone, i.e., is a monomolecular reaction in the strict sense. The first order type of mutation time curve (131, 272) may be brought about also by a bimolecular reaction with excess of one reacting partner. Thus the first step of spontaneous mutation may be partly the collision between a mutagenic metabolite and a reacting group of the gene. Such a substance could be H_2O_2, for Wyss *et al.* (313) have found that in micrococci the catalase poison NaN_3 increases the mutation rate.

The analogy of spontaneous colour changes of *Serratia* with the normally rarer mutations noted above demonstrates that both variations are of the same nature. The difference is apparently due to the frequency factor. The flagellar antigen mutations of *S. typhimurium* surely may have a similar basis. An interesting type of frequent mutation has been found by Wright (312) in a serine or glycine requiring *E. coli*. Here mass reversions to partial or full prototrophy occur in a series of steps in a medium containing glyceric acid. The apparently specific action of this acid is not yet cleared up.

Chemically induced mutation.—The mutagenic action of a large number of chemicals has been examined recently. Many of these substances have been found to be at least weakly mutagenic. Using the Sd 4 method, Demerec *et al.* (72, 74, 76) classify as strongly mutagenic: formalin, phenol, H_2O_2, α-dinitrophenol, Mn^{++}, Fe^{++}; weaker: boric, acetic, and formic acids, trinitrophenol, ethyl-, *n*-propyl-carbamate, acriflavine, caffeine, necrosine, neutral red+light; weak: NH_3, $CuSO_4$, *n*-butyl- and isoamyl-carbamate, manganese lactate, Fe^{+++}, Sn^{++}, $CeSO_4$, Co^{++}; doubtful or not mutagenic: NaOH, KOH, $HgCl_2$, lactic acid, H_2SO_4, H_3PO_4, HNO_3, HCl, $AgNO_3$, Na_2SiO_3, sodium salicylate, methyl-carbamate, neutral red in the dark, K-ferro- and

ferri-cyanide, $SnCl_4$, $CeCl_2$, $Ce(NO_3)_2$, Ni^{++}. Latarjet *et al.* (155) have revealed as mutagenic for T1-phage resistance of *E. coli:* styryl 430, desoxycholic acid, cholic acid, hypertonic NaCl, dibenzanthracene, ethyl-, isopropyl-, *n*-propyl-carbamate; as inactive: methylcholanthrene, 1,2-benzacridine-10-carbolic acid, isotonic NaCl, theobromine, sodium octanoate, sodium undecanoate, decaglycol ether of dodecanol, sodium laurate, sodium oleate, and sodium stearate. Colour sector and dwarf colony mutations in *Serratia* [Kaplan (136, 138, 141)] and T7-resistance mutations in *E. coli* B (139) are induced by erythrosine in the dark and by $NaHSO_3$ [Kaplan (146a)]. In *Fusarium*, tetrachloronitrobenzene is mutagenic [McKee (184)]. In yeast lyophilisation or alcohol medium yields "ploidy" mutations [Subramaniam *et al.* (276, 277)], but here selection is not excluded, and cytological structure seems not be to cleared up. Antiserum induces auxotrophic mutations in *Neurospora* macroconidia at 23°C. only weakly, not specifically, and not at all at 2°C. and in microconidia [Andersson-Kottö (3)]. Streptomycin is not mutagenic in *E. coli* as tested by T1-phage and streptomycin resistant mutants of a partly streptomycin resistant strain [Newcombe & Nyholm (205)]. In *Paramecium*, genetic changes of variability are induced by nitrogen mustards. They occur more rarely in the cytoplasm, mostly in the nucleus, but the complicated inheritance did not allow identification as gene or chromosomal mutations [Geckler (106)]. Investigation of the production of mutations to prototrophy in auxotrophic *Neurospora* strains by a series of different mustards shows that those with only one reactive group are also strongly mutagenic [Kirk & Westergaard (150); Stephens & Mylroie (271)]. Thus mutagenic activity of mustards cannot be due to cross linkages between chain molecules of the chromosomes [Loveless & Revell (169)]. The basis of the mutagenic action of 2-methyl-1,4-naphthachinone seems to be a little clearer. Action of this substance is inhibited by cysteine, methionine, glutathione, and Na thioglycolate, thus suggesting action on SH groups could be possible. Nevertheless the sulfhydryl poisons $HgCl_2$, $CuSO_4$, and $AgNO_3$ do not induce mutation in the tested micrococci. On the contrary, they decrease the spontaneous mutation rate [Clark *et al.* (56)].

The mutagenic action of broth media following pretreatment with ultraviolet radiation was studied further [Haas *et al.* (121)]. The active principle, probably a peroxide, is destroyed by heat, by visible light (121), and by catalase [Wyss *et al.* (313)]. The mutagenicity increases with temperature and ultraviolet dose, but decreases again at higher doses (121). After removing the bacteria from the ultraviolet-irradiated medium the increased mutation rate is maintained for some time. Thompson *et al.* (286) have demonstrated that pyruvate depresses the mutative and bactericidal activity of ultraviolet-irradiated, as well as H_2O_2-treated, medium if present during, but not after, treatment. These findings could suggest the hypothesis that the mutation and killing of cells by ultraviolet radiation is caused too by the production of a peroxidic substance in the cytoplasm which induces the effects by diffusing to the chromosomes. However, in the action spectrum of sub-

strate activation (121) wave lengths greater than 200 mμ are most effective, but no maximum at 260 mμ is present as in the action spectrum of direct irradiation of bacteria [Kaplan (144, 146)].

Secondary conditions play a role in the mutagenic action of chemicals. H_2O_2 is active only in growing, not in resting, micrococci [Wyss *et al.* (313)]. The conditions under which antiserum induces mutation of *Neurospora* cells are mentioned above (3). The dwarf colony variants of *Serratia* are induced by erythrosine mostly at low pH. This action increases with temperature corresponding to an activation energy of about 1.7 ev. The time dependence resembles a two-hit curve [Kaplan (141)]. The factors influencing the mutagenic action of $MnCl_2$ and $FeSO_4$ have been studied intensively by Demerec and co-workers with the *E. coli* Sd 4 method. The activity of $MnCl_2$ is increased by prewashing the bacteria with hypertonic NaCl, decreased with H_2O, hypotonic NaCl solution, and Mg salt. Subsequent washing does not revert the effects (74, 77). Adsorption of Mn^{++} in the cell seems to be the essential process, but the parallelism is not quite complete because of the small mutation rate but rather high adsorption at 3°C. (231). The mutagenic action of Fe^{++} is very sensitive to temperature change (74). When Sd 4 cells are treated 15 min. in 3×10^{-5} M $FeSO_4$, then diluted 1:10 and retained for 2 hr. at 37°C., a "burst" of 875×10^{-8} mutations occurred; retained at 1°C., only 143×10^{-8} occurred. Treatment at 1°C. rather than at 37°C. resulted in only 80 to 90×10^{-8} mutations. A 1-min. treatment with $FeSO_4$ yielded a mutation rate two to five times higher than the spontaneous one. A maximum is reached after $\frac{1}{2}$ to 2 hr., depending on the concentration; after this a slight decrease occurs (74).

At this moment we are not yet able to see clearly the mechanisms of the mutagenic action of chemicals. The large number and the diversity of genetically active compounds may suggest that these substances affect the network of biochemical reactions at very different points, inducing finally some metabolites which cause mutations if they touch genetic material. But the direct action of some substances on the genetic material is not impossible, for the genes may be constructed so that mutation is the only response to very different impulses, as it is true for many biological effects, e.g., nerve reactions or protein denaturation.

The problem of the relation between the mutagenic and carcinogenic processes has been stimulated by investigations of Latarjet *et al.* (155). These authors find a strict parallelism in the degree of carcinogenicity and induction of mutations to T1-phage resistance by different carbamates and anthracene derivates. The missing correlation in substances not belonging to a series of related compounds may be due to secondary characters, as permeability, adsorption, etc. Therefore, no disproof of the mutational theory of cancer can be derived from it.

A very close relation exists between killing and mutation in some cases, e.g., in experiments on the action of acriflavine on acriflavine-sensitive and -resistant *E. coli* strains [Witkin (307)]. But in resting staphylococci the

mutation rate was not increased by H_2O_2 as it was in growing cells, despite the high killing rate [Wyss *et al.* (313)].

Radiation-induced mutation.—Radiation effects are still very useful tools for exploring the submicroscopic structures of organisms. This is caused by the rather good knowledge of the time, kind, and spatial distribution of the initial step of the action of radiation, and, further, by the utility of the hit theory as a tested working hypothesis. As explained above, this theory cannot be put aside because radiation effects are shown to be "indirect" in many cases. In the last few years a number of facts have been found to indicate that after the primary physical event, the hit, a chain of further reaction steps follows. Thus the hope of early investigators to elucidate the gene structure immediately by investigating the hit process has somewhat faded. But is is probable that these post-hit reactions do not go across the whole cell but occur in the very neighborhood, or perhaps even in the interior, of the genetic material. This chain involves a transfer of the hit energy to the molecular structure that is changed in the mutation process. This transfer may be intermolecular by activated and moving molecules or radicals, or intramolecular by electron or bonding shift, etc. One of the tasks for future work is to clear up these reaction steps and so to get information about the gene structure despite or even by means of "indirectness" of hit action.

The hypothesis that ultraviolet-mutability is due to production of a mutagenic poison within the cell analogous to substrate mutagenisation is not supported by the action spectrum of the bacterial mutations obtained by direct irradiation of cells. Kaplan (144, 146) found in *Serratia* that the colour sector, dwarf colony, and sulfathiazol resistant mutations have a wave length dependence corresponding to absorption of nucleic acid, more probably desoxyribonucleic acid (DNA) than ribosenucleic acid (RNA). Thus the primary step of induction of mutations by ultraviolet light occurs very probably in the nuclear body or chromosome of this bacterium. Nevertheless the type of chemical reaction caused by it in the chromosome may be very similar to the oxidation of broth by H_2O_2 or ultraviolet light.

In many cases the mutation is induced by only one ultraviolet energy quantum, as shown by the initial linear part of the dose curve. This is found for *E. coli* B/Sd 4 and tryptophanless prototrophs [Demerec *et al.* (72, 78)]; for sector-, dwarf-, and sulfathiazol resistant mutations in *Serratia* [Kaplan (132, 144, 146)]; for *Penicilllum* [Arima (11); Kaplan (137)]; for *Neurospora* [Swanson *et al.* (278)]; and perhaps for *Glomerella* [Struble *et al.* (275)]. But there are also cases of multihit curves, e.g., the h^- to h^+ mutation of *E. coli* 15 [Kaplan (150, 145); Lieb (164, 165)]; T1-resistants of *E. coli* B/r and B [Demerec *et al.* (70, 78)]; microcolonies of *Chaetomium* [McAulay *et al.* (180)]; and inositol-less reversions of *Neurospora* [Snyder-Brown(259)]. This could be due to multinuclear cells in the cases of h^+ and T1 resistants, or perhaps to chromosomal changes in *Chaetomium.* In inositol-less *Neurospora* the nearly linear increase on a semilogarithmic scale is more indicative of selection than mutation. A distortion of the dose effect response surely played

a role also in the early zero-point mutation curve of Demerec & Latarjet (70). In these experiments a very small number of phage particles was added and consequently lysis occurred only after a period of growth that increased with the dose of radiation, and thus an increasing approach to the end point mutation rate was caused. The end point mutation rate corresponds to a two-hit curve up to 3000 erg/mm.^{2}, then a four-hit curve begins, as can be seen when the function $\log m = n\ (\log \mu + \log D)$, where n is the hit number, is plotted. Further secondary factors which may distort the dose mutation curve are the stimulation of poor growing spontaneous mutants [Bryson (42)], and the better colony formation of ultraviolet-irradiated cells of *E. coli* B on minimal than on complete medium [Witkin (310)]. A decreased mutation rate at high doses is observed when the number of survivors used for inoculation is too small [Jordan *et al.* (130)]. But this is not the same plateau or decrease of mutation rate usual in many ultraviolet experiments, e.g., with *E. coli* [Demerec *et al.* (70, 78); Newcombe & Whitehead (201)], *Serratia* [Kaplan (144, 146)], and *Neurospora* [Swanson *et al.* (278)]. With these organisms the rare mutations increase in frequency when the frequent ones decrease. This can be interpreted either by destruction of the mutated or mutating stage by further hits or by a multihit process independent of mutation but causing resistance to further mutation.

Wave lengths above 350 mμ are mutagenic at very high doses in a few organisms, such as *Chaetomium* (180) and *Serratia* [Kaplan (146a)]. In the latter bacterium, about 6 per cent colour sector mutations are induced by 10^7 erg/cm.^{2} without noticeable killing. When cells are vitally stained by photodymanic dyes, e.g., erythrosine, the sensitivity to visible light becomes high [Kaplan (134 to 137, 146a)]. In *Serratia*, dwarf colonies appear at high rates in a two-hit process, while colour sector variants arise much more slowly in a one-hit curve. At high doses, both types reach a maximum. Possibly the difference between the hit curves of dwarf colonies obtained with light and ultraviolet radiation is due to the smaller energy quanta of visible light. But it is not yet clear to what extent dwarf colonies resulting from radiation are the result of true mutation or of other processes. The influence of dying time, concentration, pH, and temperature on photodynamic effects can be interpreted in terms of permeability and adsorption of the dye. In *Penicillium* (137) and *E. coli* 15 h$^-$ (140, 145), photodynamic and ultraviolet-induced mutation processes are very similar regarding dose curve and correlation to killing. Phage resistance (139) and Sd 4 mutations [Demerec *et al.* (76)] are also induced by photodynamic action.

The dose-mutation curves for x-rays are mostly of the one-hit type, apparently without decrease at high doses. This has been demonstrated recently for the Sd 4 mutations [Anderson (4); Demerec (72)], the auxotrophs of *S. typhimurium* selected by penicillin [Plough *et al.* (213, 214, 215)], and perhaps for *Penicillium* [Arima (12)]. The concavity of the curve of ad$^-$ prototrophs of *E. coli* (4) is perhaps the result of selection. The formal target volumes of bacterial mutations are rather small, 320 Å^3 for Sd 4, 500 Å^3 for

B/r/T1 (70) compared with 11000 Å^3 in *Drosophila* (288). A study of ionisation density effects would be able to show whether this is due to real smallness of the sensitive spot (a chromosome section ?) or to small efficiency of ionisation. That mutations are induced in P^{32}-labeled cells not only by ionisation but also by transmutations of P^{32} to S^{32} is shown by Rubin (244) for *E. coli*, and by Friedrich-Freksa *et al.* (102, 103) for amoebae.

Killing of cells by radiation is similar to mutation in many respects. Therefore it may often be initiated in the genetic material as a lethal mutation. But there are differences too, and it is not yet clear whether they fall in the range of variability of mutation types, or whether they prove that killing is always or often caused by entirely different processes. Atwood (16, 17, 18) shows that in *Neurospora* lethal mutation goes parallel with conidia death at low doses only. In *Penicillium*, spore killing by ultraviolet radiation and photodynamism is a two- to three-hit process (137); in other strains (11), a one-hit; but mutation is always a one-hit process. In *Serratia*, the photodynamic cell killing needs one hit, the dwarf colony formation two hits, the colour variation one hit, and the relations of killing to mutations by both visible and ultraviolet light are different (136). Also, the reversions of mutation and death by light in *E. coli* indicate differences in their natures [Newcombe *et al.* (201)]. Bacteria are killed by radiation mostly by one-hit processes [Fram *et al.* (101); Traub *et al.* (291)]. But there are exceptions, e.g., in ultraviolet killing of *E. coli* 15 h^- precultured on broth (140) but apparently not on synthetic medium (165). Preculture of *E. coli* B and B/r in glucose medium or anaerobic broth yields x-ray killing of the multihit type, but a single-hit curve in aerobic broth [Hollaender *et al.* (123)]. Cavalli *et al.* (50, 51) have found that in bacterial cultures some of the cells are phenotypically resistant, and this resistance is identical for x-ray, ultraviolet, and mustards, and disappears after some hours.

A correlation between radiation-induced and spontaneous mutation exists in many cases. A particular streptomycin dependent strain of *E. coli* frequently mutated spontaneously and after ultraviolet radiation; whereas another was stable in both respects (72). Similar correlations exist for *E. coli* 15 h^- and h^+ (165); in *Serratia* the relation between three mutations of different frequencies is the same for spontaneous and ultraviolet-induced mutations (144). Demerec (75) has derived from this fact the picture that genes contain different numbers of mutable "side groups." However, there are also genes that are unstable to spontaneous mutation but are stable against radiation, e.g., the flocculation factor of yeast [Thorne (287)] and the lac^- in *E. coli* K 12 [Davis (60)].

Radiation-induced mutability can be influenced by secondary factors. Some authors believe that this is due to "indirectness" of the radiation effect, and is an argument against the hit theory—e.g., Bracq (34). But why should these factors not influence the primary hit-sensitive spot but only the later reactions in or around it, especially if this target is not a simple normal molecule but is instead a complicated colloidal unit? If secondary factors

do influence the primary reaction, the influencing factor would be a good tool for studying the intimate structure of the sensitive spot. Of course this target need not be identical with the gene, but is perhaps a part of it or a surrounding region. The amount of indirectness should not be overrated. Fabergé (95) points out after his experiments on the influence of temperature on chromosomal changes that a fivefold increase of sensitivity by a secondary factor corresponds to only a 1.5-fold increase of the target diameter.

Factors existing during irradiation are able to influence the hit process or the first swift steps after it. Low O_2 tension decreases the sensitivity a great deal, and increases the hit number of bacterial killing by x-rays [Hollaender *et al.* (123)], but the Sd 4 mutation of *E. coli* is depressed only a little in a pure N_2 atmosphere [Anderson (4)]. The mutation of ad^- is depressed similarly to killing (4), but selection does not seem to be excluded here. Thus the role of the indirect effect by formation of H_2O_2 or other oxidised substances seems much greater in killing than in mutation. The detection of substances (cysteine, CN^-, etc.) protecting against x-ray killing [Burnett *et al.* (44); Bracq (34)] agrees with this hypothesis. Also ultraviolet mutability is influenced by substances present during irradiation. Pyruvate [Thomson *et al.* (286)] decreases, mustards or dinitrophenol (DNP) increases it [Swanson *et al.* (278, 279)], but KCN is inactive (279). In spores of a bacillus [Mefferd *et al.* (185)] and *Penicillium* [Arima (11)], the mutation rate by ultraviolet increases by wetting and germination. The kind of preculture (123), the cell size [Magni (177)], and ploidy [Tobias (289)], but not freezing [Traub *et al.* (291)], influence the killing of bacteria or yeast by ionizing radiations.

Post-treatments are able to change only the post-hit processes and thus to indicate the nature of these reactions. Unlike pyruvate (286), which may affect hit-reactions, DNP and mustards (184) influence ultraviolet mutability after irradiation and affect mainly post-hit reactions. The post-treatment with visible light after ultraviolet radiation has been studied in color mutations of *E. coli* on tetrazolium agar [Newcombe *et al.* (201)]. The fraction of the mutations irreversible by light increases according to a multihit curve (about six hits) up to the plateau, which is maximal also for the light reversible mutations. This maximum is not exceeded even in the case that, after a first ultraviolet dose followed by photoreactivation, a second ultraviolet dose is given, and this is repeated several times.

The effect of γ-rays is photostable like that of high ultraviolet doses. No photoreactivation was indicated in the photodynamic killing and mutation effect of *Serratia* [Kaplan (146a)]. Photoreactivation of ultraviolet mutations were found in *Penicillium* [Roegner (232)], *Neurospora* [Goodgal (111); Snyder-Brown (259)], *Paramecium* [Kimball (149)], and *Serratia* [Kaplan (146a)]. The degrees of reversion of mutation and killing are usually correlated but not always (201), indicating that both effects have similar but not identical post-processes. However, the photoreactivation of killing alone is rather variable. Kelner (147, 148) has found that the reactivation spectra are different for *E. coli* and *Streptomyces griseus*. The first has a maximum at

375, the second at 436 mμ. Therefore, two chemically different structures are presumably involved.

The investigation of growing and resting cultures of *E. coli* B has revealed two types of ultraviolet killing, a one-hit type being completely photoreversible, and a multihit killing being only partly photoreversible [Novick (207)]. The vegetative cells of *Bacillus cereus* are not photoreactivatable at all. The photoreversion of ultraviolet-killed *E. coli* is independent of O_2; it increases weakly with pH, strongly with temperature [Johnson *et al.* (129); Stein & Meutzner (270)].

Also heat reactivation of ultraviolet killing (270) varies among the objects. In the B strain of *E. coli* even the x-ray killing was heat reversible, but not in B/r [Anderson (5)]. Post-treatment with $FeSO_4$ or catalase reactivates ultraviolet-killed *E. coli* too [Latterjet (156); Pratt *et al.* (220)]. A further kind of recovery from ultraviolet injury may be indicated by the much higher colony number on minimal than on complete agar with *E. coli* B [Witkin (307)], but not with *Serratia* and not after photodynamic irradiation of *E. coli* B [Kaplan (146a)].

The evidence is still too incomplete to give a clear picture of the mutation process by radiation. A few steps are revealed now out of a perhaps complicated system of reactions: the hit by a unit of radiation initiating the whole process, then an oxidative step, one or more stages (perhaps related with the latter step) reversible by light, heat, or chemicals, a photostable phase produced by further hits or a dark reaction, and stages favoured by mustards or DNP. The identity, co-ordination, and place of these reactions are uncertain. Probably they occur in or close to the mutating genetic unit, since they begin (as shown by the action spectrum), and end there.

Elective mutability.—In many cases mutation can be handled as a uniform process. But this is not correct in general, since there are different types, as gene, chromosome, and genome mutations. Microbial genetics mostly works with gene mutations as shown by the one-hit mechanism and the results of crossings. But some of the true multihit mutations may be due to chromosomal aberrations, and in *Neurospora* certain lethal mutations are demonstrated to be very probably chromosomal deficiencies [Atwood (17, 18)]. Surely chromosomal and gene mutations generally have different hit and post-hit processes (e.g., break fusion of the chromosome) and thus respond differently to treatments. But we must expect that such different responses exist also between groups of gene mutations, because of the chemical specificity of the gene structure. It seems that at least some of the following facts about elective mutability may be due to such genic individualism.

Wheeler (301) found a mutator gene in *Glomerella* that specifically increased only the spontaneous mutation plus to minus. In *Neurospora* [Tatum (284)] and in (*Salmonella typhosa*) [Bacon *et al.* (20, 21)] an elective origin of certain auxotrophic mutations by different agents, such as x-rays, ultraviolet, and mustards, was not found, while in *Ophiostoma* the change to dependence on nicotinic acid was favoured by caffeine, on methionine by ul-

traviolet, on inositol by both, and on lysine, uracil, and hypoxanthine by x-rays [Fries (105)]. Plough (215) obtained in *Salmonella typhimurium* with <35 kr of x-rays histidineless, with >35 kr cysteineless, and spontaneously prolineless mutants most frequently. In *Penicillium*, a mutation to colorless was more frequent relatively to other types by mustards, than by ultraviolet [Arima & Abe (14)]. Differences in the ultraviolet action spectrum of mutation types are observed in *Chaetomium* [McAulay *et al.* (180)]. Demerec *et al.* (72) reported that in *E. coli* many Sd 4 prototrophs, but only a few phage-resistant mutations arise by treatment with $FeCl_2$, and perhaps $MnCl_2$. In *Serratia*, more dwarf colonies than colour changes are induced by photodynamism; the reverse is true for ultraviolet and $NaHSO_3$ [Kaplan (136, 144, 146a)]. Different secondary conditions too are able to change the specific action of mutagenic agents. The proportion of mustard-induced morphological and auxotrophic mutations in *Neurospora* is varied by pressure [McElroy & de la Haba (181)], the fraction of different types of streptomycin resistant mutations in ultraviolet-irradiated *Bacillus* spores by the germination stage. In the experiments of Anderson (4), revealing differences in oxygen response of x-ray-induced Sd 4 and ad^- prototrophs, interference of selection seems not to be excluded.

Delay of mutation appearance.—The delayed appearance of the mutated character, after a few cell divisions, has been observed until now mainly in bacteria. Among the other organisms the ru°-mutation of *Chlamydomonas* is the only known case [Moewus (191)]. Three main explanations of the phenomenon are possible and apparently realised.

The first kind of delay is caused by purely physiological factors, e.g., dilution of an ergone of the parent gene (191), activation of the gene effect by increasing metabolite concentration [Davis (60)], or simple lag of the first cell fission [Newcombe & Scott (199)]. Here the clone of the mutated mother cell is transformed *in toto* to the new state. This is true in *Chlamydomonas* (191). In T1 resistant mutations of *E. coli* B three totally changed clones and one mixed with sensitive cells have been found [(199); Visconti di Modrone (295)]. Because $MnCl_2$ does not affect the lag phase, a prolongation of it cannot be the cause of delay in this case [Demerec & Flint (80)].

A second sort of retardation is mutation of only a part of the responsible genetic material causing sectored colonies. The most simple case is mutation of only one nucleus in multinuclear cells. The mutation to lac^+ of *E. coli* has been found to be due to this mechanism, since the number of nuclei can be correlated with the frequency of sector colonies [Demerec *et al.* (74)]. Nuclear segregation seems to occur also in *E. coli* 15 h^- [Lieb (164, 165)]. As Newcombe (202) has shown, the sectoring of freshly mutated lac^- cells is continued for a number of cell fissions. It is possible, after the finding of one mixed T1 resistant clone of *E. coli*, that a part of the retardation of these mutations is due to heterocaryosis too. However, the greater part seems to be physiological delay, and this may be reduced by addition of nucleate or casein hydrolysate [(72); Witkin (309)]. In the Sd mutations of *E. coli*, only

the heterocaryotic delay seems to play a role, and perhaps for this reason two mutations of the same organism have a different retardation mechanism [Demerec & Dollinger (79)]. Another kind of partial mutation is the change in only a part of the threads (monids) of a spitted chromosome. This is apparently realised in the inherited death of P^{32}-labeled *Amoeba proteus* [Friedrich-Freksa & Kaudewitz (102, 103)].

The third possible mechanism of retardation is labilisation of the genetic material, e.g., the induction of a very unstable "allel" that can mutate further. This seems realised in the colour sector mutations of *Serratia*, as is suggested by the statistics of the sector number per colony [Kaplan (144, 146)]. Perhaps this unstable state is the last unusually retarded link in the reaction chain of mutation process. Another mutation type with delayed instability seems to be the dwarf cell induction in yeast by nucleate [Constantin (59)], but here the mechanism is unknown.

Transfer and Distribution of Genetic Factors

Mendelian inheritance.—Evidence is continually accumulating to show that bacteria possess normal chromosome-like arrangements, and a Mendelian mechanism for exchange of their genetic factors. New fertile strains [Lederberg (160)] of *E. coli*, and one with abnormally high fertility [Cavalli (52)], have been found. In *Achromobacter fischeri*, frequent exchange of auxotrophic factors occurs within 15 min. on complete agar [McElroy *et al.* (182, 183)]. The fertility of *E. coli* K 12 can be lost by mutation [Maas (174)]. Besides the usual prototrophy method of selection of recombination zygotes, drugs are useful [Lederberg (157)]. The mathematical tools for bacterial linkage analyses are worked out, and with them a new, better, corrugated factor map of *E. coli* K 12 is designed [Bailey (22)]. Several factors for chloromycetin resistance are known, which are distributed over a large part of the map of *E. coli* K 12. Therefore the degree of drug resistance increases with the length of the chromosomal segment coming from the resistant parent [Cavalli (52, 54)].

The mechanism of bacterial gene recombination is analogous to a bimolecular reaction. Long times and high cell concentrations reveal a saturation effect; whereas short times and low concentrations show an induction effect perhaps caused by production of a copulation hormone. Apparently 10^4 cell collisions are needed for one prototrophic zygote [Nelson (198)]. The exchange of factors is not done by means of gametes or other stable or unstable filtrable substances, but surely by contact of cells [Davis (63); (198)]. Ultraviolet irradiation of the medium or addition of H_2O_2 or NaN_3 increases the frequency of exchange and the formation of "snakes" too. This suggests that the cell filaments are perhaps sexual stages of bacteria [Clark *et al.* (55); Wyss *et al.* (313)]. Since no appreciable number of cells with complementary auxotrophic factors in a partial prototrophic recombinant colony are found, apparently only one viable genetic type arises from a zygote [Allen (1)].

The truly heterozygous condition of the variegated colonies in a recom-

binant strain of *E. coli* K 12 is proved by single cell isolations [Zelle & Lederberg (318)]. Here the sensitivity to streptomycin or phages is dominant over resistance [Lederberg (162)]. These heterozygous diploids show abnormal segregation of the sugar factors, which is interpreted as an habitual elimination of a chromosome segment [Lederberg (158)]. In the normal K 12 strain, an abnormally high negative interference in this map region has been found by Newcombe & Nyholm (204, 205), so that the linear order here is uncertain.

A series of papers report hybrid analyses of fungi, algae, and protozoa: *Neurospora* [Emerson (88, 89); Fincham (97)]; yeast [Hartelius & Ditleoser (122); Leupold (163); Lindegren (166); Thorn (287); Winge & Roberts (302, 303)]; *Podospora* [Rizet & Engelmann (226)]; *Coprinus* [Mittwoch (188)]; *Allomyces* [Yaw *et al.* (316)]; *Chlamydomonas* [Moewus (189, 190, 191), Smith & Regnery (258)]; *Paramecium* [Preer *et al.* (221, 223)]. Normal Mendelian behaviour of the factors has been found, and partial chromosome maps have been obtained. In heterocaryons of *Aspergillus nidulans*, diploids heterozygous for five markers could be induced. In such diploid mycelia, somatic crossover happens occasionally, allowing linkage analysis without crossing [Pontecorvo & Roper (218, 239)]. Heterocaryons of *Neurospora* consisting of both mating types are self-fertilising [Groos *et al.* (119)].

An interesting view into the spatial organisation of the gene chain of the chromosome seems possible by means of the pseudoallels. Such very closely linked factors are detected for streptomycin resistance in *E. coli* [Demerec *et al.* (72); Newcombe & Nyholm (203, 204)], niacinless [Bonner (32)], suppressor of me$^-$ [Giles (109)] in *Neurospora*, biotinless in *A. nidulans* [Roper (235, 237)], and higher organisms [Komai (154)]. In general, they cause the same or similar phenotypes, and this suggests that chromosomes are subdivided in a number of segments, each of which is responsible for a certain phenotype. Differently located mutations in a segment cause the same or similar phenotypic character, act as allels in the heterozygous stage, but can be separated by crossing over [Goldschmidt (110)]. It seems possible that the 30 to 100 units being independently reproduced in *E. coli* according to Rahn's theory [Finney & Martin (100)] are identical with these "isophenic segments," not with "genes."

Extrachromosomal inheritance.—Surveys of the field are given by Sonneborn (261, 264, 265, 266). In *Paramecium*, the extrachromasomal factor kappa for the killer character was identified as visible, DNA-containing grains of 0.2 to 0.8 μ. The number of these bodies (a few hundred in strong killers) agrees well with the particle number calculated theoretically, and varied by x-ray inactivation [Preer (222)]. This inactivation is a one-hit process [Nanney (197)]. The ploidy of the gene K governs genetically the particle number [Chao (46)]. In a chloromycetin-containing medium, kappa is lost after one to two weeks [Brown (40)]. Kappa-free animals have only half the normal respiration rate [Wagtendonk *et al.* (296)]. There are three differently acting and genetically constant types of kappa particles, which may arise by mutation but never appear *de novo*. They can be present in a

mixed population in one animal [Dippell (82)]. Kappa causes *Paramecium* to excrete paramecin, a nucleic acid containing poison. One particle of paramecin is able to kill a sensitive animal [Austin (19)]. The type "mate killer" does not excrete the drug, but kills only the partners during mating [Siegel (256)]. The normal kappa particles are infectious, but only in unnaturally high concentration [Sonneborn (266)]. At present it seems to be only a question of taste whether kappa is classified as a parasite or as a normal part of the cytoplasm.

The transformations of the cytoplasmically inherited serotype of *Paramecium* are induced by ultraviolet radiation [Sonneborn & Schneller (260)] or in culture in the presence of antiserum at certain temperatures. They need a considerable number of fissions for perfection. The serotype obtained depends on certain stock specific alleles of different genes and on the environmental conditions inducing plasmotype [Beale (25)]. If the *A*-gene of stock 29 is crossed into a serotype A of stock 51, the antigen A 29 is produced instantaneously, a fact disproving the hypothesis of gene-produced, autosynthetically multiplying plasmagenes [Sonneborn (264)]. Transformation occurs in two steps, e.g., firstly from D to B, secondly to B or A or back to D [Skaar (257)]. The two partners of double animals (persisting conjugation) are both transformed to the same serotype (260).

The material basis of the mustard-induced cytoplasmically inherited depression of variability is not yet analysed [Geckler (106, 107)].

In yeast, the same small colony phenotype deficient in respiratory enzymes is due to either a single Mendelian gene [Shih-Yi-Chen *et al.* (255)] or to cytoplasmic factors [Ephrussi (92)]. This aberration can be induced by acriflavine, and it seems to be caused, perhaps reversibly, by inactivation of autosynthetic cytoplasmic particles (92, 93). Another example of cytoplasmic inheritance is the long-term adaptation to galactose. This has the appearance of a spontaneous mutation but irregular segregation in asci occurs [Mundkur (196)]. The fluctuation test reveals that an inducing as well as a selecting action of the substrate is involved [Spiegelman *et al.* (267)]. Single cell pedigree studies of the reversion of adaptation show a segregation of − cells from + cells beginning with the fifth generation. The statistics of this segregation agree with the hypothesis that about 100 free particles are distributed, that the chance for one particle to remain in the mother cell is one-half, and that one particle is sufficient to cause + type (268). For other cases of irregular ascospore segregation [Lindegren (167); Mundkur (195); Shih-Yi-Chen (254)], it seems not yet clear to what extent extramitosis [Winge & Roberts (304)], polyploidy [Roman *et al.* (234)], gene conversion (167), or cytoplasmic factors are responsible.

While all known auxotrophic mutations of *Neurospora* involve Mendelian factors [Tatum (284)], the adaptative high resistance to sulfonamide is constant during only a few vegetative transfers [Zalokar (317)]. In *Podospora* the plasmically inherited character s^S has been found. If s^S grows in contact with s, a transformation s^S to s is induced progressing from the contact point into the s^S mycelium about 1 cm. per day. This transformation also

rarely occurs spontaneously, beginning at a point and spreading through the whole s^S mycelium [Rizet *et al.* (227, 228, 229)].

Extrachromosomal inheritance has obviously a heterogeneous material basis. Besides virus-like infectious bodies such as kappa, normal visible cell structures such as cinetosomes [Lwoff (171); Sonneborn (263)], plastides and chondriosomes, are proved or in question. On the other side, certain systems of biochemical reactions may be reproduced autosynthetically, and therefore represent invisible and unstructured genetic transfer agents. In the whole system of life, the "plasmon" co-operates at an equal level with the chromosomes. Both are equally dependent on one another, and the same phenotypic differences may be due to the latter or the former. Plasma genes are gene-independent, persisting, autosynthetically reproducing things, and no indices exist until now showing that they are not pre-existing products or replicas of genes [Schultz (248)].

Infectious transformation.—The transfer of hereditary traits by filtrable substances is known only in bacteria, and best in pneumococci [Ephrussi-Taylor (94)]. A few new interesting cases have been described recently. Bilaudelle (27) reports introduction of O-antigen from *S. typhimurium* to *Shigella dysenteriae*. The transfer of characters from *Bacillus mesentericus* to *B. anthracis* [Manninger & Nógrádi (178)] is not confirmed [Tomcsik (290)]. The transformation in *Hemophilus influenzae* from R to different types of S has been analysed intensively [Alexander & Leidy (2)]. A serum factor, which is an albumin [Hotchkiss & Ephrussi-Taylor (126)] for the pneumococcal transformation, is not necessary in *Hemophilus*. Different R strains contain different amounts and types of transformable cells, which arise by spontaneous mutation. The transformation occurs without growth of the cells in only 3 min. In *E. coli*, not only serological traits are transferred but also sulfonamide resistance and sugar factors, as reported by Wyss (314) and Dianzani (81).

In *S. typhimurium*, a filtrable substance which is liberated from the cell by weak phages, and other deleterious agents, tranfers nutritional, fermentative, and drug resistant mutant characters with a maximal frequency of 10^{-5} [Zinder (320)]. Filtration shows that the substance consists of particles 0.1 to 0.3 μ in diameter. Ribo- and desoxyribonucleases, pancreatin, trypsin, and pancreatin do not affect it, contrary to other transforming principles. These facts perhaps reveal a relationship between transforming agents and the filtrable L forms of bacteria. The L-granula are often formed in the interior of swollen bacterial cells, are freed by bursting, and are able to regenerate normal bacteria [Tulasne (292, 293)]. They are serologically related to the parent bacterium but are not pathogenic [Minck & Minck (187)]. In some cases the swollen bodies develop after a cell fusion. L-granula are freed also by phage lysis of bacteria. The regenerated bacteria are often changed in hereditary characters [Klieneberger-Nobel (153)]. In the future the study of these particles may perhaps allow some interesting insight into the relation of genetic to regenerative structures, and also to the virus or provirus system of the cell and to the "plasmon."

Nuclear cytology.—The localisation of genetically important structures by means of the microscope in microbes is possible, mainly by analogy with higher organisms. Thus DNA-containing structures are generally considered to be chromosomal material. But such a simple analogy is ambiguous, especially if the structures are very small, and we may well understand that yeast cytology is not yet perfectly cleared up [Winge (305)]. In particular, it is not yet certain whether the two or four Feulgen positive granules visible during cell fission are centriols [Lindegren & Rafalko (168)] or chromosomes [DeLamater (65); Subarmaniam *et al.* (84, 85, 277)]. Reviews of data about the "nucleoids" of bacteria [Bisset (28); Delaporte (69); Piekarski (212)] result in the conclusion that these structures are at least functionally homologous with nuclei. In the electron or phase contrast microscope, they appear as weak, squeezed in, perhaps vesicular regions [Mudd *et al.* (193, 195); Stompen (273); Winkler *et al.* (306)]. Visconti di Modrone (294) has found morphological changes correlated with the growth phases. Bisset *et al.* (28, 29) believe they have discerned diploid and haploid stages, separated by nuclear reduction. After destruction of bacteria, Marshak (179) found fragments of nucleoid dimension showing a coiled structure. That nucleoids are in fact true nuclei was proved by a new structure-preserving method of dehydration and staining [DeLamater *et al.* (66, 67, 68)]. In *B. megatherium*, a filamentous prophase, a metaphase with two granular and one barlike chromosome, and a condensed telophase are clearly discerned. These findings, together with the correlation between number of nuclei and lac^+-mutated sectors found in *E. coli* [see above (74)] are indeed the first steps to a genetic cytology of bacteria.

Phenogenetics

The differences obtained experimentally by mutation concern nearly all characters which can be observed. Recently mutation to adaptative hexokinase (151), to dwarf growth caused by partial auxotrophy [Weinberg (299)] and autonomic decadence of growth [Sheng (253)] have been described, besides the ones already mentioned. In *Penicillium*, 10 main morphological mutation types are known [Arima (7, 8, 9)]. A suppressor mutation has been found in *E. coli* $B/h^-/ser^-$ acting against both defects [Witkin & Kennedy (311)]. Another in *Neurospora* is specific only against one tryptophan allel of a series [Yanofsky (315)]. Two new cases of phenocopy in bacteria have been described; one concerns the R to S change [Rogoso & Mitchell (233)], the other, citrate dependence [Ravin (225)].

The hypothesis that phenic change by mutation is primarily due to the defect of only one enzyme is upheld by other work (48). In *S. typhimurium*, 25 out of 30 induced auxotrophic mutants have a single nutrilite requirement [Plough *et al.* (215)]. Secondary effects may cause the relatively high percentage of mutants needing the same series of nutrilites in *S. typhosa* [Bacon *et al.* (21)]. Such "pleiotropy" may result from a basic step block, e.g., in the fivefold requiring *E. coli* (tyr.+phen.+trypt.+paba+*p*-hydr. benz. acid) where shikimic acid supplies the whole multiple defect [Davis (64)].

In other cases, metabolites accumulated by the first enzyme block inhibit other steps; e.g., in *E. coli*, a block of isoleucine synthesis causes accumulation of a precursor which suppresses valine synthesis [Davis (61)]. Analogous interactions have been found in methionine synthesis [Davis (62); Emerson (88)] and may be apposite in other cases [Srb (269); Teas (285)]. The temperature or pH sensitivity of auxotrophic mutants is also sometimes due to active metabolites [Maas (172, 173); Straus (274)]. The phenic mechanism of the alternative requirement in two mutants of *Neurospora* of any one of a series of amino acids [Fincham (96, 98)] is not yet quite clear. A mutual inhibition of lysine against an argininelessmutant, and of arginine against a lysineless mutant, is found in *A. nidulans*. The heterocaryon (lys^-) (arg^-) grows rhythmically because of the alternating inhibition of the two nuclei, but the heterozygous state lys^-/arg^- is prototrophic without check by the two amino acids [Pontecorvo (219)]. This indicates that the inhibitor actions occur in the cytoplasm, whereas the primary blocked reactions occur in the nucleus. A still more exact localisation of the mutation reaction step seems possible by study of pseudoallels responsible for synthetic reactions with very low rate [Pontecorvo (217, 219)]. The closely linked bi^- factors of *Aspergillus* cause auxotrophy also in the heterozygous state bi^-_1/bi^-_2, but rarely do prototrophs appear by crossover. Thus both bi^+ loci are able to work together in the biotin synthesis only when they are closely associated on the chromosome, and this suggests that the reaction step blocked by mutation occurs in the very proximity of the bi^- locus.

Resistance to drugs or phages may be due to very different mechanisms, as permeability, adsorption, competing metabolites, destruction of the drug, increased production of inhibitory substances and cytological variation [Wyss (314)]. Therefore, pleiotropic effects are not surprising [Bacon *et al.* (21); Kleczkowska (152); Szybalski (283)]. That phage resistance is due to nonabsorption can be seen by studies on isolated bacterial membranes [Weidel (298)]. Since streptomycin-dependent strains do not destroy the antibiotic, production of an antipoison may be responsible [Rubin & Steinglass (240)]. A study of cross-resistance to 13 antibiotics in a series of mutants of *E. coli*, each resistant to one of these substances, revealed that some groups of drugs behave similarly [Szybalski & Bryson (281)]. It seems that the pattern of genetic resistance is determined more by the drug than by the organism [Demerec (71, 73)]. There are two types: (*a*) full resistance is reached only by the accumulation of many mutation steps, each one of which occurs in a different locus and confers only weak resistance (penicillin, aureomycin, chloromycetin, neomycin, terramycin); (*b*) full resistance can be obtained by one step, and weaker or other types (e.g., drug dependence) occur by mutation of the same locus or a closely linked one (streptomycin) [Bryson & Demerec (41); Demerec *et al.* (71, 73, 74, 75)]; here only modifiers co-operate [Newcombe & McGregor (206)]. It is surprising that the loci of many different mutations related to streptomycin resistance are concentrated in a very small region of the chromosomal map (72, 75). This demon-

strates the complicated structure of a "gene" or better "isophenic chromosomal segment" mentioned above.

Population Genetics

Genetic changes in cultures of microorganisms appear to differ from those seen in higher organisms. However, no special unknown basic mechanisms, e.g., inheritance of acquired characters, are responsible for this, but the same causes known in the genetics of higher organisms, mainly mutation and selection. The strangeness of microbial population changes is essentially due to the "invisibility" of the individual and the high number of them even in a small culture or colony. Also the absence of a sexual process joined regularly with reproduction plays a role. Thus the character of a mass of microbes is the average result of co-operation of all individuals. Since spontaneous mutations have rates mainly about 10^{-6} to 10^{-7}, in a small, just visible colony of about 10^7 cells nearly all genetic types attainable by one mutation step are available for selecting conditions. Thus a gradual transformation of the whole culture is caused by the selective action of the environment. To elucidate the causal factors of such "adaptation," special genetic methods are necessary, especially for determination of mutation frequencies and selections rates. Useful new tools for this study are the chemostat (192, 208, 209) and the genetic marking of cells [Atwood *et al.* (15); Schneider (247)].

Analysis of population changes reveals interesting selecting factors, e.g., in *Brucella* (86). Added alanine suppresses the S type [Goodlow *et al.* (113)]. When penicillin [Braun *et al.* (39)] or D-asparagine [Goodlow *et al.* (115)] is given, alanine is excreted into the medium, and therefore the R type is favoured. On the other side, L-asparagine induces excretion of valine, and thus the S type prevails (115). This type is favoured also by a serum globulin and by Na-pyrophosphate. The latter is hindered by Mg or Mn [Cole & Braun (57, 58)]. The suppression of the S type by alanine is inhibited by pantothenate [Mika *et al.* (186)]. In some cases, virulent types are favoured by simple host passage [Gowen (117)] or injection of threonine into the host [Page *et al.* (211)]. The biological meaning of the differentiation of R and S types seems to be that the first is the more autotrophic, "plant like" stage, using simple nutritional conditions, and the second the "animal-like," parasitic, aggressive, and fastidious state [Seppilli (249 to 252)]. Selection plays an important role in the origin of prototrophs in auxotrophic strains. Sometimes prototrophs are favoured by a more complete medium, e.g., in a purineless *E. coli* [Guthrie (120)]; in other cases they are at a disadvantage there, as in *E. coli* 15 h^- [Atwood *et al.* (15)] or pantothenicless yeast [Raut (224)]. Similarly streptomycin resistant cocci are restrained in streptomycin free medium [Welsch (300)]. This does not seem to be due to a differential growth rate of sensitive and insensitive strains [Linz (170)]. The mechanism of the selective actions of drugs is not always a direct injury to the more sensitive cells but can be indirect. Bunting (43) found more white cells of *Serratia* in cultures with small amounts of detergents, although white cells are killed

more rapidly than red cells in high drug concentrations. The explanation is that the drug stimulates autolysis, and the liberated products, especially carbohydrates, favour the growth of white cells.

Development of drug resistance is due, in general, to selection of mutants by the drug. This can be seen directly by the appearance of resistant sectors in sensitive colonies growing in the presence of low streptomycin concentrations [Demerec *et al.* (74)], and is further indicated by the arising of resistant clones [Dittrich *et al.* (33, 83)]. The attainment of a higher degree of streptomycin fastness in serial cultures with inocula exposed to low rather than high drug concentrations [Gibson & Gibson (108)] probably is due to the greater variety of mutant types available for further selection. Nevertheless, the long lag period in media containing drugs suggests the occurrence of enzymatic adaptation as well as selection [Barer (23)]. Also the existence of a small mutagenic action of the chemotherapeutic agent does not seem to have been sufficiently examined.

If only mutation operates in a population, an equilibrium of types corresponding to the proportion of mutation rates is reached, as shown by the antigenic types of *S. typhimurium* [Stocker (272)]. The equilibrium is shifted when selection is important. This is observed, e.g., in *E. coli* with the chemostat [Novick & Szilard (209)]. But if growth time is long enough, new mutants arise from the old ones and the faster growing mutants overwhelm the old types. Such stepwise appearance of new mutants is observed in phage resistance of *E. coli* B (209), alanine resistant S types of *Brucella* [Braun *et al.* (35); Goodlow *et al.* (112, 114)], tyrosine independents of *Lactobacillus* [James (127, 128)], and h^+ in *E. coli* 15/h^- [Atwood *et al.* (15); Schneider (247)]. In the latter case, new, faster growing h^- mutants are produced in the earlier h^- population periodically. Each h^- type gives rise to an h^+ type overgrowing the old h^+, and thus a fluctuating equilibrium of h^+ and h^- is maintained over a long period. The phylogenetic function of this mechanism seems to be the preservation of a certain number of prototrophic cells in an auxotrophic strain under nonminimal conditions in spite of their selective disadvantage (15).

Population genetics of microbes is interesting not only for pure biology but is important also for the applied sciences such as medicine, epidemiology [Braun (36); Gowen (117, 118)], phytopathology, and industrial production of penicillin and other chemicals [Arima (6 to 14)].

LITERATURE CITED

1. Allen, G., *Microbiol. Genet. Biol.*, **3,** 5 (1950); **4,** 5, (1951)
2. Alexander, H. E., and Leidy, G., *J. Exptl. Med.*, **93,** 345 (1951)
3. Andersson-Kottö, I., *Hereditas*, **37,** 289 (1951)
4. Anderson, E. H., *Proc. Natl. Acad. Sci. U. S.*, **37,** 340 (1951)
5. Anderson, E. H., *J. Bact.*, **61,** 389 (1951)
6. Arima, K., *J. Antibiotics*, **3,** 285 (1949)
7. Arima, K., *J. Antibiotics*, **3,** 347 (1949)
8. Arima, K., *J. Antibiotics*, **3,** 445 (1949)
9. Arima, K., Nakamura, H., and Ötani, K., *J. Antibiotics*, **3,** 451 (1949)
10. Arima, K., Tanaka, K., and Masuda, K., *J. Antibiotics*, **3,** 493 (1949)
11. Arima, K., *J. Antibiotics*, **4,** 232 (1950)
12. Arima, K., *J. Antibiotics*, **4,** 277 (1950)
13. Arima, K., *J. Antibiotics*, **4,** 281 (1950)
14. Arima, K., and Abe, S., *J. Antibiotics*, **4,** 342 (1950)
15. Atwood, K. C., Schneider, L. K., and Ryan, F. J., *Proc. Natl. Acad. Sci. U. S.*, **37,** 146 (1951)
16. Atwood, K. C., and Norman, A., *Proc. Natl. Acad. Sci. U. S.*, **35,** 696 (1949)
17. Atwood, K. C., *Biol. Bull.*, **99,** 332 (1950)
18. Atwood, K. C., *Genetics*, **35,** 95 (1950)
19. Austin, M. L., *Physiol. Zoöl.*, **24,** 196 (1951)
20. Bacon, G. A., Burrows, T. W., and Yates, M., *Brit. J. Exptl. Path.*, **31,** 703 (1950)
21. Bacon, G. A., Burrows, T. W., and Yates, M., *Brit. J. Exptl. Path.*, **31,** 714 (1950)
22. Bailey, N. T. J., *Heredity*, **5,** 111 (1951)
23. Barer, G. R., *J. Gen. Microbiol.*, **5,** 1 (1950)
24. Barrat, R. W., and Tatum, E. L., *Science*, **112,** 122 (1950)
25. Beale, G. H., *Nature*, **167,** 256 (1951)
26. Bertani, G., *Genetics*, **36,** 598 (1951)
27. Billaudelle, H., *Z. Immunitätsforsch.*, **107,** 356 (1950)
28. Bisset, K. A., *The Cytology and Life History of Bacteria* (The Williams & Wilkins Co., Baltimore, Maryland, 1950)
29. Bisset, K. A., Grace, J., and Morris, E. O., *Exptl. Cell. Research*, **2,** 388 (1951)
30. Boag, I. W., *Genetics*, **36,** 281 (1951)
31. Boivin, A., *Zentr. Bakt. Parasitenk.*, **155,** 58 (1950)
32. Bonner, D. M., *Genetics*, **35,** 655 (1950)
33. Bornschein, H., Dittrich, W., and Höhne, G., *Naturwissenschaften*, **38,** 383 (1951)
34. Bracq, Z. M., *Experentia*, **7,** 11 (1951)
35. Braun, W., Goodlow, R. J., and Kraft, M., *Genetics*, **35,** 656 (1950)
36. Braun, W., *Brucellosis, Pub. Am. Assoc. Advancement Sci.*, 26 (1950)
37. Braun, W., and Howell, E., *J. Bact.*, **60,** 366 (1950)
39. Braun, W., Kraft, M., Mead, D. D., and Goodlow, R. J., *Bact. Proc.*, 52 (1951)
40. Brown, C. H., *Nature*, **166,** 527 (1950)
41. Bryson, V., and Demerec, M., *Ann. N. Y. Acad. Sci.*, **53,** 283 (1950)
42. Bryson, V., *Genetics*, **35,** 657 (1950)
43. Bunting, M. I., *J. Bact.*, **59,** 241 (1950)
44. Burnett, W. T., Stapleton, G. E., and Hollaender, A., *Federation Proc.*, **10,** 22 (1951)
46. Chao, P. K., *Microbiol. Genet. Bull.*, **3,** 70 (1950)

47. Catcheside, D. G., *Ann. Rev. Physiol.*, **12,** 47 (1950)
48. Catcheside, D. G., *Heredity*, **4,** 269 (1950)
49. Catcheside, D. G., *The Genetics of Microorganisms* (Sir Isaac Pitman & Sons, Ltd., London, England, 223pp., 1951)
50. Cavalli, L. L., and Visconti di Modrone, N., *Ricerca sci.*, **19,** 8 (1949)
51. Cavalli, L. L., and Visconti di Modrone, N., *Ricerca sci.*, **19,** 9 (1949)
52. Cavalli, L. L., *Nature*, **166,** 991 (1950)
53. Cavalli-Sforza, L. L., *Boll. ist. sieroterap. milan.*, **29** (1950)
54. Cavalli, L. L. (Personal communication); *Symposium Microbiol. Genet. Copenhagen* (1951)
55. Clark, J. B., Haas, F., Stone, W. S., and Wyss, O., *J. Bact.*, **59,** 375 (1950)
56. Clark, J. B., Wyss, O., and Stone, W. S., *Nature*, **166,** 340 (1950)
57. Cole, L. J., and Braun, W., *J. Immunol.*, **64,** 111 (1950)
58. Cole, L. J., and Braun, W., *J. Bact.*, **60,** 283 (1950)
59. Constantin, T., *Compt. rend. soc. biol.*, **144,** 1442 (1950)
60. Davis, B. D., *Genetics*, **35,** 661 (1950)
61. Davis, B. D., *J. Clin. Invest.*, **29,** 808 (1950)
62. Davis, B. D., and Mingioli, E. S., *J. Bact.*, **60,** 17 (1950)
63. Davis, B. D., *J. Bact.*, **60,** 507 (1950)
64. Davis, B. D., *J. Biol. Chem.*, **191,** 315 (1951)
65. DeLamater, E. D., *J. Bact.*, **60,** 321 (1950)
66. DeLamater, E. D., *Science*, **113,** 477 (1951)
67. DeLamater, E. D., and Hunter, M. E., *Am. J. Botany*, **38,** 659 (1951)
68. DeLamater, E. D., and Mudd, S., *Exptl. Cell. Research*, **2,** 499 (1951)
69. Delaporte, B., *Advances in Genetics*, **3,** 1 (1950)
70. Demerec, M., and Latarjet, R., *Cold Spring Harbor Symposia Quant. Biol.*, **11,** 38 (1946)
71. Demerec, M., *J. Clin. Invest.*, **28,** 891 (1949)
72. Demerec, M., Wallace, B., Witkin, E. M., and Bertani, G., *Carnegie Inst. Wash. Yearbook*, **48,** 154 (1949)
73. Demerec, M., *Trans. N. Y. Acad. Sci.*, **12,** 186 (1950)
74. Demerec, M., Witkin, E. M., Katlin, B. W., Flint, J., Belser, W. L., Dissosway, C., Kennedy, F. L., Meyer, N. C., and Schwartz, A., *Carnegie Inst. Wash. Yearbook*, **49,** 144 (1950)
75. Demerec, M., *Am. Naturalist*, **84,** 5 (1950)
76. Demerec, M., Bertani, G., and Flint, J., *Am. Naturalist*, **85,** 119 (1951)
77. Demerec, M., *Science*, **113,** 473 (1951)
78. Demerec, M., and Cahn, E., *Microbiol. Genet. Bull.*, **4,** 5 (1951)
79. Demerec, M., and Dollinger, E. I., *Microbiol. Genet. Bull.*, **4,** 6 (1951)
80. Demerec, M., and Flint, J., *Microbiol. Genet. Bull.*, **4,** 7 (1951)
81. Dianzani, M. U., *Experentia*, **6,** 332 (1950)
82. Dippell, R. von, *Heredity*, **4,** 165 (1950)
83. Dittrich, W., *Experentia*, **7,** 431 (1951)
84. Duraiswami, S., and Subramaniam, M. K., *Cellule*, **53,** 215 (1950)
85. Duraiswami, S., and Subramaniam, M. K., *Experentia*, **7,** 422 (1951)
86. Eigelsbach, H. T., Braun, W., and Herring, R. D., *J. Bact.*, **61,** 557 (1951)
87. Englesberg, E., and Stanier, R. Y., *J. Bact.*, **58,** 171 (1949)
88. Emerson, S., *Cold Spring Harbor Symposia Quant. Biol.*, **14,** 40 (1950)
89. Emerson, M. A., and Emerson, S., *Microbiol. Genet. Bull.*, **4,** 7 (1951)
92. Ephrussi, B., *Pubbl. staz. zool. Napoli*, **22,** 1 (1950)

93. Ephrussi, B., and Hottinguer, H., *Nature*, **166**, 956 (1950)
94. Ephrussi-Taylor, H., *Endeavour*, **9**, (1950)
95. Fabergé, A. C., *Genetics*, **35**, 663 (1950)
96. Fincham, J. R. S., *J. Biol. Chem.*, **182**, 61 (1950)
97. Fincham, J. R. S., *J. Genetics*, **50**, 221 (1951)
98. Fincham, J. R. S. (Personal communication); *Symposium Microbiol. Genet. Copenhagen* (1951)
100. Finney, D. J., and Martin, L., *Biometrics Bull.*, **7**, 133 (1951)
101. Fram, H., Proctor, B. E., and Dunn, C. G., *J. Bact.*, **60**, 263 (1950)
102. Friedrich-Freksa, H., and Kaudewitz, F., *Z. Elektrochem.*, **55**, 575 (1951)
103. Friedrich-Freksa, H., and Kaudewitz, F., *Microbiol. Genet. Bull.*, **5**, 7/4, 9 (1951)
104. Fries, N., *Hereditas*, **36**, 368 (1950)
105. Fries, N., *Hereditas*, **36**, 134 (1950)
106. Geckler, R. P., *Genetics*, **35**, 253 (1950)
107. Geckler, R. P., *Genetics*, **35**, 108 (1950)
108. Gibson, M. J., and Gibson, F., *Nature*, **167**, 113 (1951)
109. Giles, N. H., Jr., *Genetics*, **35**, 108 (1950)
110. Goldschmidt, R. B., *Proc. Natl. Acad. Sci. U. S.*, **36**, 365 (1950)
111. Goodgal, S. H., *Genetics*, **35**, 667 (1950)
112. Goodlow, R. J., Mika, L. A., and Braun, W., *J. Bact.*, **60**, 291 (1950)
113. Goodlow, R. J., Braun, W., and Mika, L. A., *Arch. Biochem.*, **30**, 402 (1951)
114. Goodlow, R. J., Braun, W., and Mika, L. A., *Proc. Soc. Exptl. Biol. Med.*, **76**, 786 (1951)
115. Goodlow, R. J., Tucker, L., Braun, W., and Mika, L. A., *Bact. Proc.*, 144 (1951)
116. Gottschewski, G., *Zentr. Bakt. Parasitenk.*, **155**, 31 (1950)
117. Gowen, J. W., *Scientia*, **44**, 145 (1950)
118. Gowen, J. W., *Genetics in the 20th Century* (The MacMillan Company, New York, N. Y , 401 pp , 1951)
119. Groos, S. R., *Biol. Bull.*, **99**, 331 (1950)
120. Guthrie, R., *Abstracts Soc. Am. Bact., 49th Meeting*, 15 (1949)
121. Haas, F., Clark, J. B., Wyss, O., and Stone, W. S., *Am. Naturalist*, **84**, 261 (1950)
122. Hartelius, V., and Ditleoser, E., *Nature*, **168**, 385 (1951)
123. Hollaender, A., Stapleton, G. E., and Martin, F. L., *Nature*, **167**, 103 (1951)
124. Horowitz, N. H., *Advances in Genetics*, **3**, 33 (1950)
125. Horowitz, N. H., and Mitchell, H. K., *Ann. Rev. Biochem.*, **20**, 465 (1951)
126. Hotchkiss, R. D., and Ephrussi-Taylor, H., *Federation Proc.*, **10**, (1951)
127. James, A. P., *Genetics*, **35**, 116 (1950)
128. James, A. P., *J. Bact.*, **60**, 719 (1950)
129. Johnson, F. H., Flagler, E. A., and Blum, H. F., *Proc. Soc. Exptl. Biol. Med.*, **74**, 32 (1950)
130. Jordan, W. E., Meffert, R. B., and Wyss, O., *Science*, **114**, 436 (1951)
131. Kaplan, R. W., *Z. Naturforsch.*, **2b**, 308 (1947)
132. Kaplan, R. W., *Z. Naturforsch.*, **3b**, 29 (1948)
133. Kaplan, R. W., *Zentr. Bakt. Parasitenk.*, **153**, 129 (1948)
134. Kaplan, R. W., *Naturwissenschaften*, **35**, 127 (1948)
135. Kaplan, R. W., *Nature*, **163**, 573 (1949)
136. Kaplan, R. W., *Arch. Mikrobiol.*, **15**, 152 (1949)
137. Kaplan, R. W., *Planta*, **38**, (1949)
138. Kaplan, R. W., *Naturwissenschaften*, **37**, 249, 276 (1950)
139. Kaplan, R. W., *Naturwissenschaften*, **37**, 308 (1950)

140. Kaplan, R. W., *Naturwissenschaften*, **37,** 547 (1950)
141. Kaplan, R. W., *Ber. deut. botan. ges.*, **63**(12) (1950)
142. Kaplan, R. W., *Naturwissenschaften*, **38,** 120 (1951)
143. Kaplan, R. W., *Die Umschau*, **51,** 491 (1951)
144. Kaplan, R. W., *Z- Natursforsch.*, **7b,** 291 (1952)
145. Kaplan, R. W., *Microbiol. Genet. Bull.*, **4,** 12 (1951)
146. Kaplan, R. W., *Microbiol. Genet. Bull.*, **5,** 8 (1951)
146a. Kaplan, R. W. (Unpublished data)
147. Kelner, A., *Sci. American*, 22 (May, 1951)
148. Kelner, A., *J. Gen. Physiol.*, **34,** 835 (1951)
149. Kimball, R. F., *Genetics*, **35,** 673 (1950)
150. Kirk, J., and Westergaard, M., *Nature*, **166** 1019, (1950)
151. Klein, H. P., and Doudoroff, M., *J. Bact.*, **59,** 739 (1950)
152. Kleczkowska, J., *J. Gen. Microbiol.*, **4,** 298 (1950)
153. Klieneberger-Nobel, E., *Bact. Revs.*, **15,** 77 (1951)
154. Komai, T., *Am. Naturalist*, **84,** 381 (1950)
155. Latarjet, R., Buu-Hoi, N. P., and Elias, C. A., *Pubbl. staz. zool. Napoli*, **22,** 1 (1950)
156. Latarjet, R. (Personal communication); *Symposium Microbiol. Genet. Copenhagen* (1951)
157. Lederberg, J., *J. Bact.*, **59,** 211 (1950)
158. Lederberg, J., *Genetics*, **35,** 119 (1950)
159. Lederberg, J., in *Methods in Medical Research*, **3** (The Year Book Publishers, Inc., Chicago, Ill., 1950)
160. Lederberg, J., *Science*, **114,** 68 (1951)
161. Lederberg, J., *Microbiol. Genet. Bull.*, **5,** 15 (1951)
162. Lederberg, J., *J. Bact.*, **61,** 549 (1951)
163. Leupold, U., *Compt. rend. lab. Carlsberg, Sér. physiol.*, **24,** 381 (1950)
164. Lieb, M., *Genetics*, **35,** 675 (1950)
165. Lieb, M., *Genetics* (In press)
166. Lindegren, C. C., and Lindegren, G., *Genetics*, **35,** 675 (1950)
167. Lindegren, C. C., *Yeast in Feeding, Pub. Assoc., Food Container Inst.*, 93 (1950)
168. Lindegren, C. C., and Rafalko, M. M., *Exptl. Cell. Research*, **1,** 169 (1950)
169. Loveless, A., and Revell, S., *Nature*, **165,** 938 (1949)
170. Linz, R., *Compt. rend. soc. biol.*, **145,** 146 (1951)
170a. Luria, S. E., and Delbrück, M., *Genetics*, **28,** 491–511 (1943)
171. Lwoff, A., *Problems of Morphogenesis in Ciliates* (John Wiley & Sons, Inc., New York, 1950)
172. Maas, W. K., *Genetics*, **35,** 677 (1950)
173. Maas, W. K., *Bact. Proc.*, 128 (1950)
174. Maas, W. K., *Microbiol. Genet. Bull.*, **4,** 16 (1951)
175. Macdonald, K. D., and Pontecorvo, G., *Heredity*, **4,** 278 (1950)
176. Macdonald, K. D., and Pontecorvo, G., *Microbiol. Genet. Bull.*, **2,** 10 (1950)
177. Magni, G. E. (Personal communication); *Symposium Microbiol. Genet. Copenhagen* (1951)
178. Manninger, R., and Nógrádi, A., *Experentia*, **4,** 276 (1948)
179. Marshak, A., *Proc. Natl. Acad. Sci. U. S.*, **37,** 38 (1951)
180. McAulay, A. L., Ford, J. M., and Dobie, D. L., *Heredity*, **3,** 109 (1949)
181. McElroy, W. D., and de la Haba, G., *Science*, **110,** 640 (1949)

182. McElroy, W. D., *Microbiol. Genet. Bull.*, **2,** 11 (1950)
183. McElroy, W. D., and Friedman, S., *J. Bact.*, **62,** 120 (1951)
184. McKee, R. K., *Nature*, **167, 611** (1951)
185. Mefferd, R. B., and Wyss, O., *J. Bact.*, **61,** 357 (1951)
186. Mika, L. A., Braun, W., Jr., Goodlow, R. J., and Mead, D. D., *Bact. Proc.*, 79 (May, 37–31, 1951)
187. Minck, R., and Minck, A., *Compt. rend. soc. biol.*, **145,** 927 (1951)
188. Mittwoch, U., *J. Genetics*, **50,** 202 (1951)
189. Moewus, F., *Ber. deut botan. ges.*, **54,** (45) (1936)
190. Moewus, F., *Z. Vererbungslehre*, **78,** 418, 463, 501 (1940)
191. Moewus, F., *Portugaliae Acta Biol.*, A, Goldschmidt-Vol. 161 (1949)
192. Monod, J., *Ann. inst. Pasteur*, **79,** 390 (1950)
193. Mudd, S., and Smith, A. G., *J. Bact.*, **59,** 561, 575 (1950)
194. Mudd, S., Smith, A. G., Hillier, J., and Beutner, E. H., *J. Bact.*, **60,** 635 (1950)
195. Mundkur, B. D., *Current Sci.*, **19,** 84 (1950)
196. Mundkur, B. D., *Nature*, **164,** 614 (1949)
197. Nanney, D. L., *Microbiol. Genet. Bull.*, **3,** 12 (1950)
198. Nelson, T. C., *Genetics*, **36,** 162 (1951)
199. Newcombe, H. B., and Scott, G. W., *Genetics*, **34,** 475 (1949)
200. Newcombe, H. B., *Genetics*, **35,** 682 (1950)
201. Newcombe, H. B., and Whitehead, H. A., *J. Bact.*, **61,** 243 (1951)
202. Newcombe, H. B., *Genetics*, **36,** 570 (1951) (Abstract)
203. Newcombe, H. B., and Nyholm, M. H., *Genetics*, **35,** 603 (1950)
204. Newcombe, H. B., and Nyholm, M. H., *Genetics*, **35,** 126 (1950)
205. Newcombe, H. B., and Nyholm, M. H., *Am. Naturalist*, **84,** 457 (1950)
206. Newcombe, H. B., and McGregor, J., *J. Bact.* (In press)
207. Novick, A., *Microbiol. Genet. Bull.*, **2,** 12 (1950)
208. Novick, A., and Szilard, L., *Science*, **112,** 715 (1950)
209. Novick, A., and Szilard, L., *Proc. Natl. Acad. Sci. U. S.*, **36,** 708 (1950)
210. Opatowski, J., and Christiansen, A., *Bull. Math. Biophys.*, **12,** 19 (1950)
211. Page, L. A., Goodlow, R. J., and Braun, W., *Bact. Proc.*, 52 (May 27–31, 1951)
212. Piekarski, G., *Naturwissenschaften*, **37,** 201 (1950)
213. Plough, H. H., *J. Cellular Comp. Physiol.*, **35,** 141 (1950)
214. Plough, H. H., *Genetics*, **35,** 129 (1950)
215. Plough, H. H., Young, H. N., and Grimm, M. R., *J. Bact.*, **60,** 145 (1950)
216. Pontecorvo, G., *Heredity*, **4,** 270 (1950)
217. Pontecorvo, G., *Biochem. Soc. Symp.*, **4,** 40 (Bristol, England, 1950)
218. Pontecorvo, G., and Roper, J. A., *J. Gen. Microbiol.* (In press)
219. Pontecorvo, G., *Symposia Soc. Exptl. Biol.*, 6 (1951)
220. Pratt, R., Dufrenoy, I., and Gardner, G., *J. Am. Pharm. Assoc.*, **39,** 496 (1950)
221. Preer, J. R., and Blauch, B. M., *Genetics*, **35,** 685 (1950)
222. Preer, J. R., *Genetics*, **35,** 344 (1950)
223. Preer, J. R., *Year Book Am. Phil. Soc.*, 161 (1950)
224. Raut, C., *Genetics*, **35,** 381 (1950)
225. Ravin, A. W., *Genetics*, **35,** 687 (1950)
226. Rizet, G., and Engelmann, C., *Rev. cytol. biol. végétales*, **11,** 201 (1949)
227. Rizet, G., and Delannoy, G., *Compt. rend.*, **231,** 588 (1950)
228. Rizet, G., and Sichler, G., *Compt. rend.*, **231,** 630 (1950)

229. Rizet, G., and Sichler, G., *Compt. rend.*, **231,** 719 (1950)
230. Roberts, C., *Methods Med. Research*, **3,** 37 (1950)
231. Roberts, R. B., and Aldous, E., *Cold Spring Harbor Symposia Genet.* (In press)
232. Roegner, F. R., *Univ. Wisconsin Antibiotic Rept.*, **13,** 19 (1950)
233. Rogoso, M., and Mitchell, J. A., *J. Bact.*, **59,** 303 (1950)
234. Roman, H., Hawthorne, D. C., and Douglas, H. C., *Proc. Natl. Acad. Sci. U. S.*, **37,** 79 (1951)
235. Roper, J. A., *Nature*, **166,** 956 (1950)
236. Roper, J. A., *Microbiol. Genet. Bull.*, **3,** 7 (1950)
237. Roper, J. A., *Heredity*, **5,** 157 (1951)
238. Roper, J. A., *Experentia* (In press)
239. Roper, J. A. (Personal communication); *Symposium Microbiol. Genet. Copenhagen* (1951)
240. Rubin, B. A., and Steinglass, P., *Microbiol. Genet. Bull.*, **2,** 13 (1950)
241. Rubin, B. A., *Microbiol. Genet. Bull.*, **1,** 8 (1950)
242. Rubin, B. A., *Microbiol. Genet. Bull.*, **1,** 9 (1950)
243. Rubin, B. A. *Genetics*, **35,** 688 (1950)
244. Rubin, B. A., *Genetics*, **35,** 133 (1950)
245. Ryan, F. J., in *Methods Med. Research*, **3** (The Year Book Publishers, Inc., Chicago, Ill., 1950)
246. Ryan, F. J., *Schweiz. Z. allgem. Path. u. Bakt.*, **13,** 653 (1950)
247. Schneider, L. K., *Biol. Bull.*, **99,** 331 (1950)
248. Schultz, J., *Science*, **111,** 403 (1950)
249. Seppilli, A., and Candell, A., *Boll. soc. ital. biol. sper.*, **26,** 1 (1950)
250. Seppilli, A., *Riv. biol.*, **42,** 171 (1950)
251. Seppilli, A., *Sci. med. ital.*, **1,** 338 (1950)
252. Seppilli, A., *Quaderni biol. sper.*, **7,** 1 (1950)
253. Sheng, T. C., *Genetics*, **36,** 199 (1951)
254. Shih-Yi-Chen, *Compt. rend.*, **230,** 1897 (1950)
255. Shih-Yi-Chen, Ephrussi, B., and Hottinguer, H., *Heredity*, **4,** 337 (1950)
256. Siegel, R. W., *Microbiol. Genet. Bull.*, **3,** 12 (1950)
257. Skaar, P. D., *Microbiol. Genet. Bull.*, **3,** 24 (1950)
258. Smith, G. M., and Regnery, D. C., *Proc. Natl. Acad. Sci. U. S.*, **36,** 246 (1950)
259. Snyder-Brown, J., *J. Bact.*, **62,** 163 (1951)
260. Sonneborn, T. M., and Schneller, M., *Microbiol. Genet. Bull.*, **3,** 14 (1950)
261. Sonneborn, T. M., *Bios*, **21,** 31 (1950)
262. Sonneborn, T. M., *J. Exptl. Zoöl.*, **113,** 87 (1950)
263. Sonneborn, T. M., *J. Heredity*, **41,** 222 (1950)
264. Sonneborn, T. M., *Harvey Lectures Ser. 44*, 145 (1950)
265. Sonneborn, T. M., *Centennial*, 243 (1951)
266. Sonneborn, T. M., *Sci. American*, **2,** 30 (1950)
267. Spiegelman, S., Sussman, R. R., and Pinska, E., *Proc. Natl. Acad. Sci. U. S.*, **36,** 591 (1950)
268. Spiegelman, S., DeLorenzo, W. F., and Campbell, A. M., *Proc. Natl. Acad. Sci. U. S.*, **37,** 511 (1951)
269. Srb, A. M., *Botan. Gaz.*, **111,** 470 (1950)
270. Stein, W., and Meutzner, I., *Naturwissenschaften*, **37,** 167 (1950)
271. Stevens, C. M., and Mylroie, A., *Nature*, **166,** 1019 (1950)

272. Stocker, A. D., *J. Hyg.*, **47**, 398 (1949)
273. Stompen, H., *J. Bact.*, **60**, 81 (1950)
274. Straus, B. S., *Arch. Biochem.*, **30**, 292 (1951)
275. Struble, B. B., and Keitt, G. W., *Am. J. Botany*, **37**, 565 (1950)
276. Subramaniam, M. K., and Pralada Rao, L. S., *Experentia*, **7**, 98 (1951)
277. Subramaniam, M. K., *Nature*, **168**, 427 (1951)
278. Swanson, C. P., McElroy, W. D., and Miller, H., *Proc. Natl. Acad. Sci. U. S.*, **35**, 513 (1949)
279. Swanson, C. P., and Goodgal, S. H., *Genetics*, **35**, 695 (1950)
280. Szwarc, M., and Leigh, C. H., *Nature*, **167**, 486 (1951)
281. Szybalski, W., and Bryson, V., *Microbiol. Genet. Bull.*, **5**, 10 (1951)
282. Szybalski, W., *Microbiol. Genet. Bull.*, **5**, 10 (1951)
283. Szybalski, W., *Microbiol. Genet. Bull.*, **5**, 16 (1951)
284. Tatum, E. L., *J. Cellular Comp. Physiol.*, **35**, 119 (1950)
285. Teas, H. J., *J. Bact.*, **59**, 93 (1950)
286. Thompson, T. L., Mefferd, R. B., Jr., and Wyss, O., *J. Bact.*, **62**, 39 (1951)
287. Thorne, R. S. W., *Compt. rend. lab. Carlsberg, Sér. physiol.*, **25**, 101 (1951)
288. Timofeeff-Ressovsky, N. W., Zimmer, K. G., and Delbrück, M., *Nachr. Ges. Wiss. Göttingen*, **1**, 189 (1935)
289. Tobias, C. A., *Federation Proc.*, **10**, 595 (1951)
290. Tomcsik, J., *Schweiz. Z. Path. u. Bakt.*, **12**, 489 (1949)
291. Traub, F. B., Huber, W., Brasch, A., and Friedemann, U., *Federation Proc.*, **10**, 422 (1951)
292. Tulasne, R., *Compt. rend. soc. biol.*, **144**, 1200 (1950)
293. Tulasne, R., *Compt. rend. soc. biol.*, **145**, 429 (1951)
294. Visconti di Modrone, L., and Visconti di Modrone, N., *Experentia*, **7**, 62 (1951)
295. Visconti di Modrone, N., *Microbiol. Genet. Bull.*, **5**, 16a (1951)
296. Wagtendonk, W. J. van, Zill, L. P., and Simonsen, D. H., *Proc. Indiana Acad. Sci.*, **60**, 64 (1950)
298. Weidel, W., *Z. Naturforsch.*, **6b**, 251 (1951)
299. Weinberg, E. D., *J. Infectious Diseases*, **87**, 299 (1950)
300. Welsch, M., *Compt. rend. soc. biol.*, **144**, 732 (1950)
301. Wheeler, H. E., *Am. J. Botany*, **37**, 304 (1950)
302. Winge, Ö., and Roberts, C., *Compt. rend. lab. Carlsberg, Sér. physiol.*, **25**, 35 (1950)
303. Winge, Ö., and Roberts, C., *Nature*, **166**, 1114 (1950)
304. Winge, Ö., and Roberts, C., *Nature*, **165**, 157 (1950)
305. Winge, Ö., *Compt. rend. lab. Carlsberg, Sér. Physiol.*, **25**, 85 (1951)
306. Winkler, A., Knoch, N., and König, H., *Naturwissenschaften*, **38**, 241 (1951)
307. Witkin, E. M., *Microbiol. Genet. Bull.*, **2**, 14 (1950)
308. Witkin, E. M., *Methods Med. Research*, **3** (The Year Book Publishers, Inc., Chicago, Ill., 1950)
309. Witkin, E. M., *Proc. Natl. Acad. Sci. U. S.*, **36**, 724 (1950)
310. Witkin, E. M., *Microbiol. Genet. Bull.*, **4**, 17 (1951)
311. Witkin, E. M., and Kennedy, F. L., *Am. Naturalist*, **85**, 141 (1951)
312. Wright, B. (Personal communication); *Symposium Microbiol. Genet. Copenhagen* (1951)

313. Wyss, O., Haas, F., Clark, J. B., and Stone, W. S., *J. Cellular Comp. Physiol.*, **35**, 133 (1950)
314. Wyss, O., *Ann. N. Y. Acad. Sci.*, **53**, 183 (1950)
315. Yanofsky, C., *Microbiol. Genet. Bull.*, **5**, 12 (1951)
316. Yaw, K. E., and Cutter, V. M., Jr., *Mycologia*, **43**, 156 (1951)
317. Zalokar, M., *Genetics*, **35**, 700 (1950)
318. Zelle, M. R., and Lederberg, J., *J. Bact.*, **61**, 351 (1951)
319. Zelle, M. R., *J. Bact.*, **61**, 345 (1951)
320. Zinder, N., *Microbiol. Genet. Bull.*, **5**, 13 (1951)

THE CORYNEFORM BACTERIA[1]

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Ehrlich eingestandene und begründete Unsicherheit ist besser als scheinbare Sicherheit, ohne die Angabe, worauf sie sich gründet.—Lehmann & Neumann: *Bakteriologische Diagnostik*

There are perhaps few groups of bacteria of which the typical representatives are easier to recognize and the aberrant types more numerous and more difficult to separate than those which originally constituted the genus *Corynebacterium*, and which we now may cautiously call the group of coryneform bacteria.

When first created by Lehmann & Neumann (1) to give room for the diphtheria bacillus and later for a few like organisms, the genus formed a delightfully simple group of pathogenic or at least animal-parasitic bacteria characterized by an irregular, often clublike cell shape, absence of endospores, immotility, gram-positive reaction, often irregular staining, nonacid-fastness, and a predominantly aerobic mode of life. During nearly six decades several groups of organisms with some of the original distinctive features have been discovered, and borderline cases have been found to link up with other groups; all this has made the outlines of the genus so fluid that it becomes somewhat hard to blame Conn (2) for protesting against "the misuse of the term *Corynebacterium*." The existence of "transitional" or "borderline" forms may be a grievous impediment to group separation and nomenclature but may help us to visualize, albeit in a vague and tenuous way, certain trends that indicate a system based on phylogeny, following the lines laid down by Kluyver & van Niel (3) and Stanier & van Niel (4).

The Group of Coryneform Bacteria and Its Distribution in Nature

As coryneform bacteria in a wider sense we may regard the following groups:

(*A*) A "nucleus" of predominantly animal-parasitic bacteria representing the original genus *Corynebacterium* (*sensu stricto*) [Lehmann & Neumann (1)]. Besides occurring in several well-defined human and animal diseases, these organisms have most frequently been found on the nasal, pharyngeal, and other mucous membranes and the skin, also in the intestine (5) and in the udders of healthy cows (6, 7). Closely allied to these is a small group of thermoduric bacteria with a distinctive metabolism that is usually given generic rank as *Microbacterium* [Orla-Jensen (8)]. The common source

[1] The survey for the literature pertaining to this review was concluded in November, 1951.

of isolation of the latter is milk and dairy utensils, but their absence from aseptically drawn milk (9) suggests that their natural habitat is not the udder, but possibly the animal skin or intestine.

(*B*) A large group of saprophytic (largely soil-inhabiting) or plant-pathogenetic bacteria of similar morphology, but sometimes motile. The first inclusion of such an organism in *Corynebacterium* seems due to Honing (10), but the first general extension was made by Kisskalt & Berend (11), who included several soil and water forms in the genus. Jensen (12, 13) found similar organisms abundantly represented in soil; this discovery was confirmed by Topping (14), Ørskov (15), and Krasilnikov (16, 17), as well as by Clark (18) and Lochhead (19), who also found them, although relatively less frequently, in the rhizosphere of plants. Clark (20) has presented evidence that several species of *Cellulomonas* probably belong here. Jensen (13) transferred certain plant pathogens to the genus, and a further extension in this direction was made by Dowson (21) and Skaptason & Burkholder (22). Water forms have been mentioned (11, 23) but have appeared rare until Wood (24) reported their frequent isolation (apparently mud inhabitants) from sea water and shark flesh. Conn & Dimmick (25) suggested the generic name *Arthrobacter* for some members of the soil group characterized by a more varied cell morphology, weaker gram-reaction of young cells, stronger proteolytic activity, weaker acid formation, and simpler nutritive demands than the parasitic corynebacteria. The proposed genus includes *A. globiforme*, one of the commonest soil inhabitants (26), and perhaps also Krasilnikov's (17) genus *Mycococcus* which may be identical with some of Conn's "coccus-forming rods." Since the differences are largely quantitative, the present writer thinks that the genus *Arthrobacter* should be kept in abeyance until more information on the rest of the saprophytic coryneforms has accumulated.

These two groups, which we may provisionally call "*Corynebacterium sensu lato*," are separated by somewhat flimsy borderlines from a few other genera: (*a*) *Propionibacterium*, morphologically similar to *Corynebacterium*, but having a strictly fermentative metabolism, (*b*) *Mycobacterium*, strongly oxidative and generally acid-fast but bordering upon *Corynebacterium* through strains in which the last character is only slightly accentuated, (*c*) *Nocardia* and *Actinomyces* which produce a more or less well-defined mycelium in early stages of their life-cycle.

Morphology and Reproduction

Cell shape.—The "classical" appearance of the *Corynebacteria*—as irregular cells varying from long, club- or wedge-shaped, occasionally branched rods, to short, almost coccoid elements—often recurs in the related groups but is not always distinct in some recognized members of *Corynebacterium*, e.g., *C. pseudodiphtheriticum*. Bisset (27, 28) has interpreted the club-shape as an artefact produced by fixation and staining by routine methods, but

this view is hardly tenable, as Hewitt (29) has shown that the club shape is also seen in living specimens studied by phase-contrast microscopy. Nevertheless the pronounced club shape is often missing, and it is more correct to speak of a tendency to club-formation as characteristic of the group.

Cell inclusions.—The well-known stainable bodies known as "metachromatic granules" have always been held characteristic of the type species although their presence depends on nutritional conditions. Although absent in some other parasitic forms, e.g. *C. pseudodiphtheriticum*, they occur in several members of the saprophytic group according to Clark & Mitchell (30). Their interpretation as a kind of reproductive structure (gonidia?) by Gróh (31) remains unproven, but the fact that they can be seen in living specimens, as shown by Hewitt (29), seems to disprove Bisset's (27, 28) view of them as artefacts.

Reproduction.—A constant feature that runs through the whole group is the mechanism, or one of the mechanisms, of cell division: the "snapping" growth that results in the characteristic angular arrangement of the cells which was first shown by Ørskov (32) to occur also in *Mycobacterium* and in *Nocardia* at certain stages succeeding the formation of mycelium. The sudden snapping of the rods may be an artefact caused by physical stress in the medium under a coverslip, as contended by Bisset (28), but the ease with which the typical configuration of the cells can be observed on open agar plates (13, 32) shows plainly that Ørskov's "angular growth" is not a fortuitous occurrence but a distinctive character of the coryneform bacteria.

Besides binary fission, another mode of reproduction by simultaneous multiple division of long cells into short elements has been described by Gróh (31), Nyberg (33), and Bisset (27, 28). Here we arrive at what appears to be an important point in the morphological makeup of the whole group. Bisset (27, 28) claims that the individual organisms of *Corynebacterium* and *Mycobacterium* are multicellular bacteria that divide either by simple binary fission or by multiple division into individual cells [evidently identical with Gróh's (31) "Glieder"] that regenerate the original multicellular bacterium. Bisset & Moore (34) also studied *Actinomyces* and a number of soil "diphtheroids" for which they suggested the name *Jensenia;* it is not clear whether this is not a synonym for *Arthrobacter.* These were found to be unicellular organisms with occasional branching or formation of an early unstable mycelium, as in *Nocardia,* which was found multicellular like *Corynebacterium* and *Mycobacterium.*

The multicellularity of the (parasitic) corynebacteria was confirmed by phase-contrast observations on living cells by Hewitt (29) who, however, pointed out a striking degree of variation in *C. diphtheriae:* the *mitis* and *gravis* strains showed few partitions but the *intermedius*-strains so many as to make the individual organisms appear like strings of cocci. This, together with Hewitt's conclusion that "presumably each cellular compartment on regeneration contains a complete genetic structure for the transmission of

the strain," offers a natural explanation for the formation of coccoid elements in later stages of development in saprophytic corynebacteria (13, 25) as well as *Nocardia*.

Branching, which is fairly frequent in the soil forms, may be seen also in the parasitic group, not only in stained (27, 31, 33) but also in living specimens (29). Although of a transient nature, as pointed out by Bisset (27, 28), it indicates a tendency to mycelium-formation which reaches a somewhat higher degree in the *Arthrobacter* (or *Jensenia*) group (25, 35, 36), becomes obvious in *Actinomyces* and *Nocardia*, and reaches its full development in *Streptomyces* and *Micromonospora*.

Motility.—Nonmotility has been regarded as one of the cardinal features of *Corynebacterium*, and no member of the parasitic group has yet been shown to possess true motility. A different state of affairs meets us in the saprophytic and plant-pathogenic group; the first statement on motility comes from Honing (10) on an irregular, gram-positive rod with one to four polar flagella, isolated from tobacco. Whether this organism showed the characteristic mode of cell division is unknown, but no such uncertainty exists in the case of an organism isolated from contaminated blood by Friedenreich (37); this showed typical snapping growth and angular cell arrangement, and was motile by a single polar flagellum. The coryneform soil bacteria previously studied by the present writer (13) were not then seen to exhibit true locomotion, but some of these organisms were later found by Kluyver (38) to be actively motile when grown in shake cultures. Since then, the reports on motility in coryneform bacteria have increased rapidly. Topping (14) found polar flagella in numerous soil organisms that otherwise resembled true corynebacteria, and pointed out the apparent need of recognizing motile species in *Corynebacterium* as well as *Nocardia*. Similar soil organisms were encountered by Ørskov (15), and Taylor (36) observed motility (type of flagellation not stated) in many strains of *Bact.* (*Arthrobacter*) *globiforme*. Jensen (39) isolated a motile cellulose-decomposing soil *Corynebacterium*. Wood (24) found occasional motility in marine corynebacteria, and stated that this property was readily lost on subcultivation. Dowson (21) transferred two motile, gram-positive plant pathogens from *Phytomonas* to *Corynebacterium*, although it is not clear whether their mode of cell division is that of the latter genus. Most surprising of all is perhaps the observation of Clark & Carr (40) on motility in coryneform bacteria hitherto regarded as constantly nonmotile, e.g., *C. fimi* and *C. michiganense*. The fact that motility is best detected by special methods of cultivation, like semi-solid agar (40) or shaking (38), raises the question as to whether similar methods might not reveal motility in the animal-parasitic group.

The existence of motile coryneform bacteria becomes less surprising in view of the fact that motility occurs, rarely but indisputably, in *Streptococcus* and *Sarcina*, and extends into the closely related genus *Nocardia*, as shown by Topping (14) and Ørskov (15) in numerous branching soil organisms of the *Nocardia*-type. Species of *Actinomyces* and of parasitic

Nocardia, e.g., the numerous strains tested by von Magnus (41), have always been described as nonmotile, but exceptions seem to exist. Colien (42) found a motile, coccoid stage in a *Nocardia*-like organism isolated from a human throat, and the present writer (unpublished observation) noticed the arising of motile cocci in "*Actinomyces*" *dermatonotus* [Bull (43)], a sheep parasite that obviously belongs to *Nocardia*.

As long as motility is not demonstrated in the parasitic corynebacteria, we must regard *Corynebacterium* (*sensu stricto*) as "genuinely" and the group of soil inhabitants and plant pathogens as "incidentally" nonmotile (3).

Carbon Metabolism

Studies on the fermentation reactions of *C. diphtheriae* and parasitic forms associated with it are legion, and have shown a very wide spectrum of reactions ranging from fermentations of numerous carbon compounds with formation of acid but no gas (fermentative metabolism) to a purely oxidative metabolism with little or no acid formation. Examples of the first are the type species and numerous animal pathogens and nonpathogenic parasites (44, 45, 46) as well as the *Microbacterium*-group (8, 9, 47), and of the second group *C. pseudodiphtheriticum*, the udder corynebacteria (6, 7), and *C. equi* (13, 44, 48).

By comparison, our knowledge of the nature of the fermentation processes is remarkably scanty. De Wolff (49) first showed, by qualitative tests only, that *C. diphtheriae* ferments glucose and mannose, and an unidentified skin diphtheroid also sucrose, with formation of formic, acetic, propionic, lactic, and succinic acid, in addition to small amounts of ethyl alcohol. In experiments with resting cells of *C. diphtheriae*, Fujita & Kodama (50) found anaerobic fermentation of glucose to formic and lactic acid plus small amounts of acetic and succinic acid; the two former products could aerobically be respired to carbon dioxide and water. Tasman & Brandwijk (51) confirmed the qualitative observations of de Wolff and postulated a double mechanism of glucose dissimilation: a splitting, with phosphorylation, of the glucose-chain in two three-carbon fractions resulting in lactic acid and subsequently propionic acid fermentation, and a splitting without phosphorylation into a two-carbon and a four-carbon chain giving rise to other compounds. They also observed a marked Pasteur effect of oxygen on the sugar metabolism and later (52) confirmed the observation of Fujita & Kodama on oxidative respiration of the organic acids produced in sugar fermentation. *C. diphtheriae* thus possesses a typical combination of fermentative and oxidative metabolism. Olsen (15) found that certain intestinal corynebacteria were biochemically typical heterofermentative lactic acid bacteria producing lactic and acetic acid from glucose. From organisms of this biochemical type there is only a short step to *Microbacterium* in which lactic acid is the chief fermentation product (47), and to certain small-mycelial strains of *Nocardia* studied by von Magnus (41), which biochemically were true homofermentative lactic acid bacteria, and for which the generic name *Lactomyces* was sug-

gested. The sugar metabolism of the microaerophilic *C. acnes* is a typical propionic acid fermentation according to Douglas & Gunter (53).

Even more sketchy is our knowledge of the respiration process in the soil- and plant-pathogenic corynebacteria. Conn & Dimmick (25) emphasize that although some acid formation may take place (13), the soil organisms that constitute their genus *Arthrobacter* are as a whole weak sugar fermenters of a strongly oxidative character, in contrast to the parasitic group. This is certainly true of the prototype of the group, *A. globiforme*, and is possibly a general rule, but not one without exception. Several cellulose-decomposing corynebacteria (20, 39) as well as some plant pathogens, e.g., *C. sepedonicum* [Snieszko & Bonde (54)], ferment sugar vigorously, but very little is known about the fermentation products. In an unidentified cellulose-decomposing organism, Jensen & Swaby (55) found formic and acetic acid, with small amounts of lactic acid. Wood (24) has recently pointed out a remarkable tendency to variation in the fermentation pattern of marine corynebacteria to an extent that seems to make this property unreliable for taxonomic purposes. This might suggest a segregation of genetic factors during the reproduction by multiple-cell fragmentation [cf. the variability in agar-decomposing *Streptomyces* which was thought by Stanier (56) to be connected with the conidia formation in the aerial mycelium].

Nitrogen Metabolism

Forms of nitrogen required.—The nitrogen requirement of *C. diphtheriae* has been set out clearly by Mueller (57), who showed that only aspartic acid or aspartic and glutamic acid, sometimes interchangeable, are unconditionally essential for detectable growth of exacting strains; cystine is essential as a source of sulphur only, and may be replaced by other reduced sulphur compounds. For optimum growth, exacting strains require in addition one or more of the following monoamino acids: glycine, leucine, valine, tryptophan, and methionine. Certain nonexacting strains of Braun & Mündel (58) produce detectable growth merely with aspartic acid and cystine. The nitrogen requirements of the animal pathogens and the nonpathogenic "diphtheroids" appear not to have been worked out in detail. Conn & Dimmick (25) found eleven representatives of this group unable to grow "on any synthetic medium investigated, a fact which indicates their need of some organic form of nitrogen, or accessory growth factors, or both." (Note that the term "synthetic medium" is obviously used in a sense different from that of Mueller.) The *Microbacterium*-group seems rather more exacting than *C. diphtheriae;* Doetsch & Pelczar (59) were able to cultivate only one species, *M. lacticum*, in a medium which besides accessory factors contained 18 amino acids representing the hydrolysis products of casein; it remains uncertain whether these were all essential. The parasitic corynebacteria thus demand nitrogen in an organic (amino acid) form of more or less complicated character. The plant pathogens are somewhat less exacting and utilize ammonia in the presence of accessory factors, according to Starr (60).

In the saprophytic (soil) group we meet a more varied picture. Several of these, e.g. *C. helvolum, filamentosum,* and *simplex,* as well as *Arthrobacter globiforme,* are definitely nonexacting and grow well with nitrate, ammonia, or urea (13, 25). Numerous coryneform soil bacteria, however, behave differently. A number of strains resembling *Microbacterium liquefaciens* [Orla-Jensen (8)] were found to require organic nitrogen alone or accompanied by accessory factors (13). Topping (14) studied 113 strains of motile and nonmotile soil corynebacteria, of which a large proportion were unable to utilize nitrate or ammonia, and Lochhead & Chase (61) found that their coryneform soil bacteria (nonmotile, gram-positive rods) represented a wide spectrum of exactingness ranging from nitrate-utilizing strains, through others requiring amino acids, to still others that demanded unknown constituents of yeast, or yeast and soil, extract, as nitrogen source or accessory factors. *C. fimi* and an unnamed cellulose-decomposing *Corynebacterium* ["V6" (39)] studied by Fåhraeus (62) were unable to utilize nitrate but would grow on glucose when provided with ammonia; amino acids, however, improved the growth, and still other amino acids seemed essential (as constituents of the cellulose-decomposing enzyme?) for growth on cellulose. The range of variation thus appears considerably wider than in the parasitic group, and even the segregation of the nonexacting organisms in a separate genus *Arthrobacter* would leave us with a large number of species to be placed elsewhere.

Proteolysis.—Proteolytic activity is usually considered slight or absent in the parasitic species, mainly on the basis of their failure to liquefy gelatin and coagulated serum or to digest milk. Conn & Dimmick (25) emphasize this point in their definition of *Arthrobacter,* which like numerous other soil forms shows vigorous action in gelatin and milk (13). This general rule, however, admits of several exceptions. Firstly, certain parasitic species do liquefy gelatin, e.g. *C. pyogenes* and a serological group of animal-pathogenic corynebacteria of human origin studied by Poulsen (45), who saw the same phenomenon incidentally in other groups. Secondly, absence of gelatin liquefaction does not necessarily imply inactivity towards other proteins. Thus de Wolff (49) found vigorous breakdown of milk proteins by *C. pseudodiphtheriticum;* Jensen (13) observed a slight proteolysis in milk by the same organism, and some strains of *C. renale* appear to have similar effects in milk (44) and peptone (63). Also several members of the *Microbacterium*-group liquefy gelatin, and a small number digest casein (8, 9). The difference in proteolytic activity between the parasitic and the saprophytic group thus seems to be one of degree rather than of kind and hardly reliable for generic separation.

Urease activity and deamination.—The ability to hydrolyze urea is widespread among the parasitic corynebacteria although absent in *C. diphtheriae* (45, 46); it is characteristic of *C. renale* and *C. ovis,* according to Lovell & Harvey (13). These authors found urease activity lacking in *C. equi* and *C. pyogenes* which, on the other hand, showed strong deaminating activity towards glutamine and arginine, respectively.

Decomposition of purine compounds.—Certain saprophytic corynebacteria are able to metabolize purine derivatives. Krebs & Eggleston (64) described a soil organism named *C. ureafaciens* (generally resembling *C. helvolum* but reported to be acid-fast) of strongly oxidative character and forming urea from all compounds containing the grouping $=N-\overset{\|}{C}-N=$. Lara (65) has found strains of the *C. filamentosum-simplex*-group able to utilize uracil and thymine as sole source of carbon and nitrogen.

Growth Factor Requirements

The growth factor requirements of most coryneform bacteria are comparatively little known apart from the general fact that parasitic species "demand complex media." *C. diphtheriae*, however, has long been known to grow and produce toxin in media of defined composition. Mueller (57) has defined the unconditionally demanded factors of exacting strains for normal growth as pimelic and nicotinic acid, and in some cases also pantothenic acid or β-alanine. The nonexacting strains of Braun & Mündel (58) seem to demand no accessory factors for mere detectable growth, and Mueller (57) points out that *C. diphtheriae* apparently comprises a spectrum of varying requirements. *Microbacterium lacticum*, which according to Doetsch & Pelczar (59) requires pantothenic acid as a truly essential factor, besides other B-vitamins for additional growth stimulation, appears comparable to *C. diphtheriae*, and the extreme degree of exactingness is shown by certain organisms of unknown origin, which were found by Svendsen *et al.* (66) to resemble *Hemophilus* in their demand for coenzyme I (V-factor) and hemin (X-factor), in addition to ascitic fluid for optimum growth.

Plant-pathogenic species, according to Starr (60), demand thiamin and biotin, in some cases nicotinic acid or nicotinic acid and pantothenic acid, and thus appear no less (rather more) exacting than *C. diphtheriae*.

The range of variation is even wider among the soil forms. Types of the "*Arthrobacter*"-group require no accessory factors [Conn & Dimmick (25)], but numerous others studied by Lochhead & Chase (61) demand growth factors of the B-vitamin complex, or unknown substances. One of the few items of precise information is due to Fåhraeus (62), who studied two cellulose-decomposing corynebacteria: *C. fimi* produced detectable growth in simple glucose or cellulose medium plus ammonia or amino-nitrogen, but considerable stimulation was produced by thiamin and sometimes biotin; an unnamed strain, "Vb" [Jensen & Swaby (55)], required both vitamins. The spectrum in the saprophytic and plant-pathogenic corynebacteria thus extends from no requirements to a degree of exactingness that equals or exceeds that of *C. diphtheriae*. To place the nonexacting species in a separate genus *Arthrobacter* would seem somewhat arbitrary, not least since Knight & Proom (67) have shown the existence of a similar spectrum in a group of bacteria so obviously natural as the aerobic mesophilic spore-formers.

Taxonomic Considerations (A Tentative Phylogenetic Scheme)

It is only with hesitation that one attempts a "natural" classification of bacteria as a whole, when it is borne in mind that our bacterial "species," as van Niel (68) points out, conform more to the Linnean-species concept than to species in the sense of phylogenetic classification of higher plants and animals. Nevertheless the existence of a kind of developmental cycle in the *Actinomycetaceae* which in many points resemble the coryneform bacteria, and the recurrence of certain phases of this cycle in the coryneform bacteria suggest that such an attempt may be ventured on the basis of the principles of Kluyver & van Niel (3) and Stanier & van Niel (4). Also the abundance of "transitional" forms and the arising of mutants that skip the arbitrary lines of group separation give us a clear impression of organisms belonging "naturally" together, even though we may not yet venture to predict which forms represent the ancestral and which the descendant types.

In the widest sense we may define the coryneform bacteria as nonspore-forming bacteria of irregular rod shape varying from branching cells sometimes approaching small mycelia down to almost coccoid elements, the central majority being rods composed of one or several "individual cells" (Bisset), "compartments" (Hewitt) or "Glieder" (Gróh). They are gram-positive (to varying degrees), not acid-fast or only slightly so. They are incidentally motile, reproducing by binary fission or multiple fragmentation, the former accompanied by the characteristic "angular growth" that recurs in organisms clearly recognizable as actinomycetes. Their energy metabolism ranges from purely oxidative to partly or purely fermentative, and the biochemical mechanisms for lactic and propionic acid formation are present.

The saprophytic soil organisms of the "*Arthrobacter*"-type appear as a primitive and versatile group that embodies the above mentioned characters, and from which other groups may be derived by the loss or the intensification of these characters. In this group the energy metabolism is almost wholly oxidative, synthetic ability is well developed (growth with inorganic nitrogen without accessory factors), and proteolytic power is considerable. Partial loss of synthetic ability leads us to the soil saprophytes that require organic nitrogen or growth factors, and further partial loss of oxidative power to organisms like the cellulose-decomposing corynebacteria with marked acid formation. We may call this "Group O."

From this group we may derive a "Group 1*a*" (*Corynebacterium sensu stricto*) of organisms adapted to parasitic existence. Here the incidental motility is lost, other morphological characters are retained, but proteolytic and synthetic abilities are lessened (demand for organic nitrogen or for organic nitrogen and growth compounds). The metabolism remains oxidative in some species, while in others it becomes partly fermentative through partial loss of oxidative power. *Microbacterium* seems to have its natural place here. Speck (47) claimed that the group should be placed close to *Lactobacillus* and *Propionibacterium*, but even if this is conceded, it does not justify its re-

moval from *Corynebacterium*, because *Microbacterium* differs from the former in several essential biochemical features (presence of catalase, high Q_{O_2}, sensitivity to cyanide), as shown by Hansen (69), and has little in common

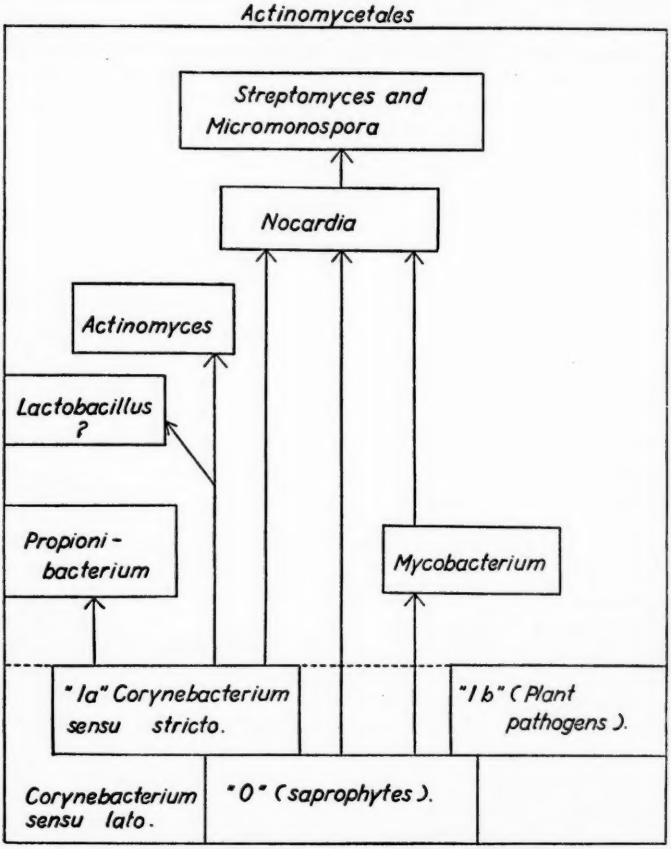

FIG. 1. Tentative scheme of relationships between coryneform bacteria and genera of *Actinomycetales*.

with the latter except its morphology which it shares with *Corynebacterium*. In the present writer's opinion, which is also held by Abd-el-Malek & Gibson (9), *Microbacterium* may be regarded as a group of thermoduric strains of *Corynebacterium* rather than as a separate genus.

Close to Group O we find a section of organisms ("Group 1*b*") adapted

to parasitism in plants; in these the incidental motility and the oxidative or partially fermentative metabolism remain, while synthetic ability is lessened.

These three groups thus constitute *Corynebacterium sensu lato*. In other groups that may be derived from them, more profound morphological or biochemical changes become evident. Proceeding from Group 1*a* we arrive at two groups where the metabolism has become purely fermentative. One of these is *Propionibacterium* which has morphology and lack of synthetic ability in common with *Corynebacterium* (*sensu stricto*), but whose katabolism consists in propionic acid fermentation. The inclusion of *C. acnes* in this genus by Douglas & Gunter (53) seems justified, and it would appear desirable to study the carbohydrate metabolism of other microaerophilic corynebacteria from this point of view. The second is *Actinomyces*, in which the slight and abortive branching in *Corynebacterium* has developed into formation of a fragile mycelium; its fermentative mechanism does not appear to have been studied, but a connecting link with *Corynebacterium* seems represented by "*Lactobacillus*" *bifidus*, whose sugar metabolism is a heterofermentative lactic acid fermentation, as shown by Orla-Jensen (70) and Olsen (5), and whose morphology (branching and angular growth) led these authors probably correctly to suggest its transfer to *Actinomyces* or *Corynebacterium*. The observation of Norris *et al.* (71) that *L. bifidus* seems to produce mutants similar to *L. acidophilus* suggests that we might look here for the origin of *Lactobacillus*. Also the serological studies of Slack *et al.* (72) suggest some kinship between *Actinomyces*, *Corynebacterium*, and *Lactobacillus*.

In Group O (or 1*a*) we may also find the origin of *Mycobacterium* where the metabolism has become purely oxidative and is accompanied by the synthesis of more or less waxy material, while proteolytic activity, incidental motility and the tendency to form clavate cells are lost. That *Mycobacterium* and *Cornebacterium* border closely upon each other is shown by the existence of several partially acid-fast forms, e.g., *C. equi*, whose metabolism is markedly oxidative, and whose acid-fastness *in vivo* may approach that of the tubercle bacillus (48); indeed this species might probably with as much right be placed in *Mycobacterium* (13). Prévôt's (73) classification of the *Actinomycetales* primarily on the basis of acid-fastness thus seems to represent a view as extremist as that of Krasilnikov (17), who discards acid-fastness as a diagnostic feature and includes the corynebacteria with *Mycobacterium*.

From *Mycobacterium* and *Corynebacterium* there is a very gradual transition to *Nocardia*, where mycelium formation [sometimes to an imperceptible extent, as pointed out by Umbreit (74)] is succeeded by fragmentation into rods and cocci, where acid-fastness varies in both parasitic (41) and saprophytic species (74), where some saprophytic forms are motile (14, 15), and some parasites show fermentative metabolism and even pure lactic acid fermentation (41). Proteolytic power sometimes exists, but is not combined with acid-fastness (41, 74). The tenuousness of the borderline is further illustrated by the occasional arising in saprophytic corynebacteria of mutants

of *Nocardia*-like character (13) and the loss of mycelial growth in strains of the latter group (32).

Concluding Remarks

A large part of the nonspore-forming, gram-positive bacteria may thus be arranged in a rather continuous sequence ranging from *Corynebacterium* (*sensu lato*) to *Nocardia* and thence to *Streptomyces* and *Micromonospora*, the whole sequence constituting the order *Actinomycetales*. Their connection with other gram-positive rods (*Butyribacterium*, *Kurthia*, *Bacillus*) as well as with the cocci and the gram-negative bacteria remains obscure. An observation by Wood (75) on snapping cell division in *Pseudomonas* suggests the possibility that this genus, and possibly *Rhizobium*, may have its origin in the corynebacteria, perhaps among the motile plant pathogens. The gram-negative character of *Pseudomonas* and *Rhizobium* would imply the loss of ability to accumulate ribonucleic acid—a change that appears imaginable in view of the sometimes weak gram-reaction in young cells of the *Arthrobacter*-group, as pointed out by Conn & Dimmick (25).

LITERATURE CITED

1. Lehmann, K. B., and Neumann, R. O., *Bakteriologische Diagnostik*, 1. Aufl., (J. F. Lehmanns Verlag, Munich, 448 pp., 1896)
2. Conn, H. J., *J. Bact.*, **54**, 10 (1947)
3. Kluyver, A. J., and van Niel, C. B., *Zentr. Bakt. Parasitenk.*, [II]**94**, 369–403 (1936)
4. Stanier, R. Y., and van Niel, C. B., *J. Bact.*, **42**, 437–66 (1941)
5. Olsen, E., *Studies on the Intestinal Flora of Infants* (Doctoral thesis, Royal Vet. Agr. College, Copenhagen, 1949)
6. Steck, W., *Zentr. Bakt. Parasitenk.*, [I]**124**, 227–36 (1932)
7. Bendixen, H. C., *Z. Infektionskrankh. parasit. Krankh. Hyg. Haustiere*, **43**, 106–25 (1933)
8. Orla-Jensen, S., *Kgl. Danske Videnskab. Selskabs. Skrifter*, [8]**5**(2), 81–197 (1919)
9. Abd-el-Malek, Y., and Gibson, T., *J. Dairy Sci.* (In press)
10. Honing, J. A., *Mededeel. Deli Proefsta. Medan*, **7**, 223–53 (1912)
11. Kisskalt, K., and Berend, E., *Zentr. Bakt. Parasitenk*, [I]**81**, 444–47 (1918)
12. Jensen, H. L., *Proc. Linnean Soc. N.S.W.*, **58**, 181–85 (1933)
13. Jensen, H. L., *Proc. Linnean Soc. N.S.W.*, **59**, 1–61 (1934)
14. Topping, L., *Zentr. Bakt. Parasitenk*, [II]**97**, 291–304 (1937)
15. Ørskov, J., *Zentr. Bakt. Parasitenk.*, [II]**98**, 344–57 (1938)
16. Krasilnikov, N. A., *Zentr. Bakt. Parasitenk.*, [II]**90**, 428–34 (1934)
17. Krasilnikov, N. A., *Repts. Acad. Sci., USSR*, 328 pp. (1938)
18. Clark, F. E., *Trans. Kansas Acad. Sci.*, **43**, 75–84 (1940)
19. Lochhead, A. G., *Can. J. Research*, [C]**18**, 42–53 (1940)
20. Clark, F. E., *Soil Sci. Soc. Am. Proc.*, **15**, 180–82 (1951)
21. Dowson, W. J., *Brit. Mycol. Soc. Trans.*, **25**, 311–14 (1942)
22. Skaptason, J. B., and Burkholder, P. W., *Phytopathology*, **32**, 439–41 (1942)
23. Brussoff, A., *Zentr. Bakt. Parasitenk.*, [II]**90**, 162–68 (1934)
24. Wood, E. J. F., *Australian J. Marine Freshwater Research*, **1**, 129–38 (1950)
25. Conn, H. J., and Dimmick, I., *J. Bact.*, **54**, 291–303 (1947)
26. Conn, H. J., *Bact. Revs.*, **12**, 257–73 (1948)
27. Bisset, K. A., *J. Gen. Microbiol.*, **3**, 93–96 (1949)
28. Bisset, K. A., *The Cytology and Life History of Bacteria* (E. & S. Livingstone, Edinburgh, Scotland, 136 pp., 1950)
29. Hewitt, L. F., *J. Gen. Microbiol.*, **5**, 287–92 (1951)
30. Clark, F. E., and Mitchell, R. B., *J. Bact.*, **44**, 529–32 (1944)
31. Gróh, E., *Zentr. Bakt. Parasitenk.*, [I]**141**, 209–29 (1938)
32. Ørskov, J., *Investigations into the Morphology of the Ray Fungi* (Doctoral thesis, Univ. of Copenhagen, 1923)
33. Nyberg, C., *Zentr. Bakt. Parasitenk.*, [I]**149**, 26–33 (1942)
34. Bisset, K. A., and Moore, *J. Gen. Microbiol.*, **3**, 387–91 (1949)
35. Taylor, C. B., and Lochhead, A. G., *Can. J. Research*, [C]**15**, 340–47 (1937)
36. Taylor, C. B., *Soil Sci.*, **46**, 307–21 (1938)
37. Friedenreich, V., *The Thomsen Hemagglutination Phenomenon* (Doctoral thesis, Univ. of Copenhagen, 1930)
38. Kluyver, A. J. (Personal communication)
39. Jensen, H. L., *Proc. Linnean Soc. N.S.W.*, **65**, 543–56 (1940)
40. Clark, F. E., and Carr, P. H., *J. Bact.*, **61**, 1–6 (1951)
41. von Magnus, R., *Acta Path. Microbiol. Scand.*, **25**, 11–32 (1947)

42. Colien, F. E., *J. Bact.*, **30,** 301–22 (1935)
43. Bull, L. B., *Australian J. Exptl. Biol. Med. Sci.*, **6,** 301–14 (1929)
44. Merchant, J. A., *J. Bact.*, **30,** 95–116 (1935)
45. Poulsen, J. P. V., *Studier over Korynebakteriernes Systematik* (Doctoral thesis, Univ. of Copenhagen, 1944)
46. Welsch, M., and Thibault, J., *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **14,** 193–213 (1948)
47. Speck, M. L., *J. Dairy Sci.*, **25,** 533–43 (1943)
48. Bendixen, H. C., and Jepsen, A., *Abstracts Scand. Vet. 5th Meeting*, Sect. 1, 1–33 (1939)
49. de Wolff, H. H., *Biochemische Eigenschappen van de Diphtherie- en van de Pseudodiphtheriebacterie* (Doctoral thesis, Univ. of Utrecht, 1927)
50. Fujita, A., and Kodama, T., *Biochem. Z.*, **271,** 185–98 (1934)
51. Tasman, A., and Brandwijk, A. C., *J. Infectious Diseases*, **63,** 10–20 (1938)
52. Tasman, A., and Brandwijk, A. C., *J. Infectious Diseases*, **67,** 282–91 (1940)
53. Douglas, H. C., and Gunter, S. E., *J. Bact.*, **52,** 15–23 (1946)
54. Snieszko, S. F., and Bonde, R., *Phytopathology*, **33,** 1032–44 (1943)
55. Jensen, H. L., and Swaby, R. J., *Proc. Linnean Soc. N.S.W.*, **66,** 98–106 (1941)
56. Stanier, R. J., *J. Bact.*, **44,** 555–70 (1942)
57. Mueller, J. H., *Bact. Revs.*, **4,** 97–134 (1940)
58. Braun, H., and Mündel, F., *Zentr. Bakt. Parasitenk.*, [I]**112,** 347–54 (1929)
59. Doetsch, R. N., and Pelczar, M. J., *J. Bact.*, **57,** 61–62 (1949)
60. Starr, M. P., *J. Bact.*, **57,** 253–58 (1949)
61. Lochhead, A. G., and Chase, F. E., *Soil Sci.*, **55,** 185–95 (1943)
62. Fåhraeus, G. (Personal communication)
63. Lovell, R., and Harvey, D. G., *J. Gen. Microbiol.*, **4,** 493–500 (1950)
64. Krebs, H. A., and Eggleston, L. V., *Enzymologia*, **7,** 310–20 (1939)
65. Lara, F. S. (Personal communication)
66. Svendsen, M., Amilie, L., and Henriksen, L. D., *J. Bact.*, **53,** 755–58 (1947)
67. Knight, B. C. J. G., and Proom, H., *J. Gen. Microbiol.*, **4,** 508–38 (1950)
68. van Niel, C. B., *Cold Spring Harbor Symposia Quant. Biol.*, **11,** 285–301 (1946)
69. Hansen, P. A., *Zentr. Bakt. Parasitenk*, [II]**98,** 289–97 (1938)
70. Orla-Jensen, S., *Kgl. Danske Videnskab. Selskab. Biol. Skrifter*, **2**(3), 1–144 (1943)
71. Norris, R. F., Flanders, T., Tomarelli, R. M., and György, P., *J. Bact.*, **60,** 681–96 (1950)
72. Slack, J. M., Ludwig, E. H., Bird, H. H., and Canby, C. M., *J. Bact.*, **61,** 721–35 (1951)
73. Prévôt, A. R., *Ann. inst. Pasteur*, **72,** 2–11 (1946)
74. Umbreit, W. W., *J. Bact.*, **38,** 73–89 (1939)
75. Wood, E. J. F., *Proc. Linnean Soc. N.S.W.*, **75,** 195–213 (1950)

THE HUMAN RICKETTSIOSES[1]

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The great impetus given laboratory studies of the rickettsias during World War II was reflected in the review of the field in 1947 by Topping & Shepard (171), though there was only incidental mention of epidemiology. Military needs have continued to influence phases of research on some rickettsioses. There has been a concomitant acceleration of studies on other problems, both basic and applied. Considerable work has been done on the nature and classification of the organisms, their occurrence in livestock, and on methods of prophylaxis and treatment of rickettsial diseases. The startling discovery that body lice in Korea and Japan have developed resistance to 1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl)-ethane (DDT), like flies and other insects elsewhere, has radically altered thinking on vector-control measures.

It has been possible in the space allotted to cover only a small part of these advances with documentation that will provide the reader with further pertinent references. There have necessarily been omissions including the entire and important field of animal rickettsioses not transmissible to man.

GROWTH, METABOLISM, AND CHEMICAL COMPOSITION

Growth.—Growth characteristics of *Coxiella burnetii* in embryonated eggs have been examined by Ormsbee (112), who measured rickettsial growth quantitatively by means of the complement-fixation test. The organisms exhibited lag and exponential growth phases, followed by a period of slower growth. No further rickettsial growth occurred in embryos, dead of the infection, which were subsequently incubated at 30° to 34°C. Rabinowitz, Aschner & Grossowicz (139) reported that *Rickettsia prowazekii* may multiply in chick embryos previously killed by cold, but this was not confirmed by Ito *et al.* (67). That the complement-fixing value of a suspension of living *C. burnetii* can be used as a measure of the infectiousness of the suspension is indicated from the work by Ormsbee, Lackman, & Pickens (113). They found a predictable relationship between complement-fixation value, number of infectious doses, and mean death time of the infected chick embryos.

In a related investigation (114), terramycin, aureomycin, and chloramphenicol delayed rickettsial growth in proportion to the logarithm of the dose of antibiotic. The slopes of the curves of the first two were closely similar, and differed significantly from that for chloramphenicol; a similar

[1] The survey of the literature pertaining to this review was completed in February, 1952.

logarithmic relationship between antibiotic dose and prolongation of chick-embryo life is indicated by Smadel, Jackson & Ley (155). The effectiveness against *C. burnetii* of terramycin, aureomycin, and chloramphenicol decreased in that order. Ormsbee & Pickens (114) also showed that detectible rickettsial growth was delayed by low levels of antibiotic. However, when growth was measurable, it proceeded at a rate indistinguishable from that in untreated controls. This was not true in the higher dose ranges in which the growth rate never equaled that of the controls.

Metabolism.—Bovarnick & Snyder (20) demonstrated oxygen uptake and carbon dioxide output by purified suspensions of living typhus rickettsias (*R. prowazekii* and *Rickettsia typhi*) in the presense of succinate, pyruvate, or glutamate as substrate. Greatest activity was obtained with glutamate; with this substrate the oxygen uptake was proportional to the concentration of rickettsias as measured by the 50 per cent death point in mice. Freezing and thawing caused increased oxygen uptake with succinate but a decreased uptake with pyruvate and glutamate. The authors suggest that the succinate effect may be governed by changes in membrane permeability. Glucose and lactate were not utilized by their rickettsial preparations either aerobically or anaerobically. No oxygen uptake was found (19) with fumarate, malate, oxaloacetate, citrate, butyrate, or any amino acids except glutamate. A low oxygen uptake was caused by α-ketoglutarate.

Further investigation (19) demonstrated the presence of a transaminase and indicated that the oxidation of glutamate proceeds via the citric acid cycle with aspartate as a byproduct resulting from the transaminase reaction, and that the organisms are impermeable to the dicarboxylic acid intermediates in the reaction chain.

The complete impermeability of typhus rickettsias to the dicarboxylic acid intermediates is questioned by Wisseman *et al.* (180) who reported oxygen uptake by suspensions of *R. typhi* in the presence of α-ketoglutarate, succinate, fumarate, malate, oxaloacetate, and pyruvate. Comparable preparations of normal chick-embryo yolk-sac tissue were negative. They likewise found that rickettsial suspensions in the presence of glutamate accumulate α-ketoglutarate, succinate, oxaloacetate, and pyruvate, either spontaneously or in the presence of selective inhibitors. No evidence was obtained for the participation of the tricarboxylic acid cycle except for a small oxygen uptake of questionable significance with cis-aconitate. Their inability to demonstrate the entire citric acid cycle may have been due to the loss of essential coenzymes during purification of the suspensions, although Bovarnick & Snyder (20) found that the addition of adenosine-triphosphate, cozymase, hexosediphosphate, magnesium, and manganese did not stimulate utilization of glucose.

Only suggestive evidence for the formation of high energy phosphate bonds has been obtained. Oxygen uptake was stimulated by the addition of inorganic phosphate and magnesium (19, 181) and, under certain condi-

tions, by manganese (181). Wisseman *et al.* (181) also demonstrated adenosine triphosphatase activity in their preparations, although the possibility of this enzyme being a contaminant has not yet been excluded.

In summary, it can be said that the existence of most of the reactions involved in the citric acid cycle has been demonstrated for typhus rickettsias. The problem of rickettsial permeability to various substrates has not been settled, and the existence of phosphorylating mechanisms has not been established.

Similar investigations on the metabolism of *C. burnetii* by Ormsbee (112a) demonstrated that this organism will actively metabolize succinate, pyruvate, α-ketoglutarate, and oxaloacetate. No oxygen uptake resulted from the addition of fumarate, malate, citrate, glucose, glucose-6-phosphate, glycerol, ethyl alcohol, aspartate, or glutamate to purified suspensions. The oxidation of succinate by both *C. burnetii* and *R. prowazekii* was enhanced three- to five-fold by the addition of excess cytochrome-*c* to the reaction mixture. Oxygen uptake by *C. burnetii* in the presence of α-ketoglutarate and oxaloacetate was likewise enhanced by the addition of cytochrome-*c*. The possibility that cytochrome-*c* was washed out of the organisms during purification is under investigation.

Detailed descriptions have been given of methods of preparing purified rickettsial suspensions, the composition of various suspending media, and the effect of various mixtures in stabilizing rickettsial metabolic activity (21, 181). It appears that the best media for purification and stabilization are those high in potassium, low in sodium, low in over-all inorganic ion concentration with the balance of the osmotic requirement being supplied by sucrose, and with glutamate and serum albumin added as stabilizing agents.

Inhibition of metabolism.—Wisseman *et al.* (181) reported that aureomycin, chloromycetin, terramycin, streptomycin, *p*-aminobenzoic acid (PAB), penicillin G, *p*-nitrobenzyl-penicillin, subtilin, or nitroacridine 3582 did not cause significant inhibition of oxygen uptake when added to purified suspensions of *R. typhi* with glutamate as substrate. Significant inhibition of oxygen uptake under these conditions was obtained with arsenite, selenite, fluoride, malonate, and cyanide, but not with azide.

In this connection, Loomis (91) found that aureomycin and azide specifically inhibited phosphorylation without inhibiting oxygen uptake in a mitochondrial test system and suggested that aureomycin acts by inhibiting aerobic phosphorylation. Van Meter & Oleson (176), using rat-liver homogenates, found that aureomycin inhibited oxygen uptake rapidly in the absence of citrate but slowly in its presence. They suggest that the mode of action may be a blocking of part of the Krebs cycle. It is not now possible to interpret the results obtained with *R. typhi* and aureomycin in the light of these findings, since neither phosphorylation nor citrate utilization has been demonstrated in this organism.

Snyder & Davis (162) have reported that *p*-hydroxybenzoic acid competitively reverses the rickettsiostatic effect of *p*-aminobenzoic acid on *R. typhi*, *R. prowazekii*, and *Dermacentroxenus rickettsii* growing in the chick yolk sac.

Chemical composition.—The composition of *R. prowazekii* which had been subjected to ether extraction and repeated washing has been reported (34, 35) in percentages as follows: nitrogen, 12.2; phosphorus, 0.93; lipids, 13.2 including 9.5 acetone-soluble and 3.1 phospholipids; desoxyribonucleic acid (DNA) 2 to 2.5. Amino acids identified were tyrosine, tryptophan, and arginine. Ribonucleic acid (RNA) was not found. Tovarnickij, Krontovskaja & Ceburkina (172) in a study of rickettsial preparations which had not been ether extracted reported the percentage composition of *R. prowazekii* to be: protein, 30.2 (equivalent to approximately 4.9 nitrogen); lipid, 46.6 including 29.7 neutral fat and 15.8 phospholipid; nucleic acid (Feulgen-positive), 12.0.

These results may well be somewhat inaccurate, since ether-extraction of typhus rickettsias releases large amounts of soluble antigen [see (171)], and also extensive washing may well have caused loss of cell constituents including RNA (144). Moreover, the method of nucleic acid extraction used by Tovarnickij, Krontovskaja & Ceburkina (172) is sufficient to bring about considerable hydrolysis of RNA to nucleotides.

Ris & Fox (144) have demonstrated the presence of RNA and DNA in *R. prowazekii*. Ribonuclease treatment or washing with physiological saline resulted in a marked decrease in pyronine stainability of formalin-fixed rickettsias. DNA was demonstrated in washed rickettsias by staining with methyl green and photographing at 630 mμ, and by means of the Feulgen reaction on unwashed rickettsias.

The nucleic acid composition of ether extracted, purified suspensions of *C. burnetii* has been examined by Smith & Stoker (160). Their procedure of producing very pure suspensions may also have resulted in the loss of cellular components including a significant proportion of the RNA present. The nucleic composition was reported to be 9.7 per cent DNA and about 4.3 per cent RNA. Different preparations gave constant DNA but different RNA values. Their figures for mean molar values of adenine, guanine, cytosine, and thymine in *C. burnetii* were not significantly different from those found for the chick; 5-methyl cytosine was not detected in the rickettsial preparations, while it was present to the extent of 4.3 per cent in the embryo preparations.

The foregoing data indicate that rickettsias are similar to bacteria in that they possess both RNA and DNA and are different from most viruses which possess only one nucleic acid, a possible exception being influenza. This conclusion is not in agreement with the findings of Callot & Vendrely (24) who reported that treatment of *R. typhi* with desoxyribonuclease decreased stainability with Giemsa stain but that ribonuclease treatment did not. Here again it may have been that RNA was washed out of the cells.

The following amino acids were identified chromatographically in an acid hydrolysate of *C. burnetii* (160): alanine, arginine, aspartic acids, cystine, glutamic acid, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, valine, and a trace of threonine. No tyrosine was found, in contrast to its identification in *R. prowazekii* (34).

EPIDEMIOLOGIC AND BIOLOGIC ASPECTS

Typhus Group

Epidemic typhus.—Epidemic typhus is transmitted from man to man by the body louse, the rickettsias in infected louse feces presumably gaining entrance to the body through abrasions in the skin. Inhalation of dried infected louse feces may also be responsible for some cases, since *R. prowazekii* in such material retains its virulence for long periods and since there is evidence indicating that certain laboratory and hospital cases have resulted through air-borne infection (149).

Man generally is considered the reservoir of the disease, and no proved strains have been recovered from any other vertebrate. Recent strong evidence that recrudescences of the infection actually occur (see Brill's disease, next section) and that these cases are capable of infecting lice has strengthened an earlier explanation for the mechanism by which man may act as an interepidemic reservoir of classical typhus. Other mechanisms that have been suggested are contamination of patients' clothing or bedding with infected louse feces (15), in which viable organisms may persist for long periods of time (166), ingestion of infectious feces by clean lice (137), and a once popular hypothesis that classic strains may result from adaptation of the murine strain to the cycle man-louse-man (100). As these authors and Murray & Snyder (111) have since pointed out, there is no experimental evidence to support this last hypothesis and, in fact, the results of recent investigations oppose it. For example, Freeman *et al.* (48, 49) made serologic studies of typhus in Mexico and Peru. Their studies indicated that in Mexico classic epidemic typhus was more common than murine typhus, that both types existed side by side, and that in two isolated outbreaks investigated, both early and late cases were of the classic epidemic type. In Peru these authors detected infections of epidemic typhus only, indicating that murine typhus was rare or absent and thus was probably seldom, if ever, a source of typhus outbreaks in that country. The availability of more reliable methods of differentiating *R. prowazekii* and *R. typhi* now permit the accumulation of good laboratory evidence for or against the moot question of transformation of one type into the other.

That murine typhus may produce epidemics through a concomitant man-louse-man cycle as suggested by Castañeda (27) and others, appears possible, but its actual occurrence still lacks conclusive proof. Liu (89), as well as Silva Goytia (153), recovered strains from body lice in institutional

epidemics that they considered were initiated from murine sources. Human body lice have been experimentally infected with *R. typhi* (52, 111), but no change in the character of the agent occurred. Moreover, multiplication in the guts of the lice of both this organism and *R. prowazekii* was confirmed; both infections caused death of some lice in four to eight days. Fleas also have been infected with epidemic strains [see (78, 100)]. Adaptation of human louse colonies to maintenance on rabbits, and improvements in membrane techniques of feeding, have facilitated such investigations (52, 164).

The recent discovery of DDT-resistant lice on North Korean prisoners of war and Tokyo vagrants has added to the sudden general concern regarding insecticide-resistant arthropods, a concern culminating in a number of symposia and conferences on this subject, and brought to focus by the Cincinnati Conference, December 8 and 9, 1951, under sponsorship of the National Research Council. Alternative methods of vector control are urgently needed and under scrutiny.

Brill's disease.—Brill's disease, first clinically described in 1898, was the name given to typhus-like illnesses occurring in Europeans who had immigrated to the northeastern coastal cities of the United States. Subsequent to 1946 it has been recognized in Switzerland, France, England, Portugal and in Yugoslavia [see (110)]. A detailed history of this disease has been recounted by Murray *et al.* (108). Zinsser (185) long ago advanced the novel hypothesis that Brill's disease is a recrudescence of epidemic typhus in louse-free persons immigrating from endemic areas of southeastern Europe some years previously.

Employing later refined techniques, the etiologic basis of Zinsser's thesis was confirmed by Murray *et al.* (108, 111) who identified conclusively as *R. prowazekii* strains recovered from seven nonlousy patients with Brill's disease. Moreover, the serums of these patients were positive for epidemic typhus in specific complement-fixation tests. The recovery of *R. prowazekii* in clean laboratory-bred body lice fed on the patients emphasizes the epidemiologic potentialities of this kind of typhus. Despite the etiologic and epidemiologic evidence, the authors believe that final proof of Zinsser's hypothesis requires demonstration that the disease is caused by *R. prowazekii* persisting in the individual's body and not recently introduced in some way from the outside. These workers (108) isolated rickettsias by feeding lice on patients between the fifth and eighth days of the disease, but were unable to repeat this on others after the 8th day. Attempted isolation in animals was less successful, although some strains were recovered by inoculating patient's blood into cotton rats or guinea pigs. Identification of the Brill's strains is described in detail by Murray & Snyder (111) who used virulence and cross-immunity tests in weanling white mice as critical criteria to confirm a differentiation from murine strains by the complement-fixation test.

More recently Murray and co-workers (110) studied 26 cases of Brill's disease in Yugoslavia, thus demonstrating its presence in a region where

typhus epidemics have occurred, a necessary corollary if this disease is actually a recrudescence of typhus. The typhus etiology of these cases was confirmed by specific complement-fixation and rickettsial agglutination tests. Absence of louse transmission, lack of secondary familial cases, definite history of a previous typhus attack, short clinical course, and negative Weil-Felix reaction were used as the basis of a diagnosis of Brill's disease. A rash was found in only about half the patients. None of the patients had received typhus vaccine at any time.

The serologic pattern of the complement-fixation or rickettsial agglutination tests in Brill's disease closely resembles that obtained with serums from patients ill with epidemic typhus (59, 104, 108, 111). In the Yugoslavian studies the rickettsial agglutination titers between the 12th and 20th day after onset of illness were 1:800 or higher in 23 of the cases and 1:400 or greater in the other three cases. The complement-fixation titers in all of the cases were four-to sixfold higher than the murine titers. On the other hand, the Weil-Felix reaction with *Proteus* OX19 was negative in all 26 cases. None developed a titer of 1:160 or higher, and in 25 cases the maximum titers were below 1:40. Similar observations were made by Murray *et al.* (108) on the 14 cases studied in the United States, except that three of these latter developed Weil-Felix titers of 1:160 or more. Others also have observed positive Weil-Felix reactions in some cases of Brill's disease.

The observation by some investigators (74, 182) that latent infection may be set off by some unrelated incitant such as shock or other infections may have significance here. A similar mechanism was noted by Kostrzewski (79) as causing some relapses in trench fever.

Murine typhus.—Though the important source of human infection is still considered to be the rat flea, *Xenopsylla cheopsis*, the principal means by which man acquires murine typhus, is still a matter of conjecture. Human infections have been postulated to result from rubbing infected flea feces into the skin, from ingestion of food contaminated by infected flea feces or rat urine, or from air-borne dust contaminated with these excreta. Experiments with volunteers showed that murine typhus can be contracted by ingestion of viable *R. typhi* (134). Recent observations of Pomalez-Lebron (135) and Worth & Rickard (184) with experimentally infected white and wild rats indicate that urine of infected rats may be an unimportant source of the disease. Rickard's observation (142) that *R. typhi* in infected flea feces exposed under certain natural conditions became nonviable within three to four days suggests the need for further studies along this line. Rapid drying of such material and maintenance free from moisture may possibly occur in nature and appears to prolong the infectiousness (14).

Present evidence suggests that murine typhus is transmitted among rodents by their fleas, lice, and mites [see (78)]. Such transmission by fleas and lice is well founded, but evidence of transmission of the infection by mites is conflicting. The tropical rat mite, *Bdellonyssus bacoti*, was accused

as a vector species on the basis of earlier experimental studies and later reports of its natural infection in China with *R. typhi* (90). However, other investigators have indicated that this mite is at best an inefficient vector and is often incapable of experimental transmission (138, 183). Recently, Gispen (56) demonstrated natural infection with *R. typhi* of a trombiculid mite, *Euschöngastia indica* (Hirst), taken from Javanese rats. Its vector status was not determined. This mite was observed in abundance on domestic rats in Manila, P. I., suggesting its role in maintenance of murine typhus [see (128)]. From another mite, *Laelaps echidninus*, taken from domestic rats in Buenos Aires, Manso Sato & Pletneff (96) isolated an unidentified rickettsia which behaved like *R. typhi* in animals and egg culture. However, negative attempts to infect this mite experimentally were earlier reported (101). Kalra & Rao's (75) report of natural infection with a murine strain in a tick species, *Boophilus australis*, should be confirmed.

Epidemiological evidence points to the domestic rat as the principal reservoir of *R. typhi* in the United States, but field and house mice and the domestic cat also have been naturally infected. A wide variety of rodents and other small domestic and wild animals are susceptible to experimental infection (105, 125). The presence of typhus antibodies detected in serological tests has suggested natural infection in other species.

The complement-fixation test with specific rickettsial antigens has been widely used in surveys for detection of past infection with murine typhus in rodents (22, 61, 62, 135). The rickettsial agglutination test, on the other hand, appears to be unsatisfactory for conducting such surveys, since this test was found by Rickard (141) to be considerably less accurate than the complement-fixation test in examination of serums from known infected rodents. Employing the complement-fixation test, Morlan, Hill & Schubert (105) reported typhus antibodies in eleven native mammals and one bird, the bluejay. These results should be confirmed, since Rickard & Worth (143) have shown the necessity of establishing for each animal species the dilution at which the complement-fixation test can be regarded as specific. In the surveys by the other authors, tests of large numbers of domestic cats and hens were negative, indicating that if these animals are naturally infected, the prevalence of demonstrable antibodies is not very high. The cat flea (*Ctenocephalides felis*) and the chicken flea (*Echidnophaga gallinacea*) both have been found naturally infected with *R. typhi* (see 78), and the cat flea has been circumstantially incriminated as a source of human infections (9). Evidence is accumulating that local incidence of human cases may be reduced by control measures against domestic rat ectoparasites (106).

Spotted Fever Group

The spotted fever group of rickettsial diseases consists of American spotted fever, which occurs in various parts of the Western Hemisphere, and diseases of closely related etiology which are prevalent in the Mediterranean littoral, Africa, India, Australia, and possibly Russia. While ticks have been

the accepted natural vectors heretofore, the most recently recognized disease of this group, rickettsialpox, is transmitted by parasitic mites. This complicates the classification of the group by vectors.

American spotted fever.—American spotted fever, includes Rocky Mountain spotted fever of the United States and Canada, Brazilian spotted fever, Tobia fever of Colombia, and Mexican spotted fever. These diseases represent a single entity as indicated by cross immunity in laboratory animals, clinical evidence, and protection tests with vaccines (117). Their identity is also indicated by recent studies employing specific serological tests (131, 146). There is little information on tick vectors in addition to that reviewed by Kohls (78). In Brazil, Magalhães (94) recently reaffirmed his opinion of the domiciliary role of the bed bug, a view not shared by Travassos (175). The vectors of the disease in Mexico are considered to be *Rhipicephalus sanguineus* in the north and northwest sections, and probably *Amblyomma cajennense* in the east coastal region (23). The various medical and epidemiologic aspects of these diseases are covered in detail by Parker & Oliphant (117). Recently, de Rodaniche & Rodaniche (145) have reported a case of spotted fever occurring on the Isthmus of Panama from which *Rickettsia rickettsii* was isolated.

R. rickettsii has been recovered (117a) from the tick, *Haemaphysalis leporis-palustris*, by direct inoculation of chicken eggs with tick suspensions; addition of penicillin or streptomycin served to eliminate contaminating bacteria. This method suggests a means of obtaining certain mild strains of this rickettsia from this and other tick sources which may not be recoverable by the usual method of guinea pig inoculation.

Fièvre boutonneuse.—Specific complement-fixation tests (132) support the belief that *Rickettsia conorii* is a specifically distinct infectious agent within the spotted fever group. Fièvre boutonneuse, first described by Conor & Bruch in Tunis in 1910, is now known in most countries of the Mediterranean basin. It is probable that similar infections reported in Kenya, Belgian Congo, and other regions in equatorial Africa are the same disease. South African tick-bite fever, which is widespread in South Africa and Southern Rhodesia, was considered by Pijper & Crocker (130) to be distinct from fièvre boutonneuse on clinical and vector grounds. However, specific serologic studies by Plotz (131) and laboratory evidence of other workers (50) indicate that the two diseases are identical. Additional specific or varietal names proposed for these strains do not appear warranted at present.

In the Mediterranean area the infection is a domiciliary problem and occurs on premises harboring dogs and infected *R. sanguineus* ticks, the chief vector there of this disease. On the other hand, in South Africa, dogs are not considered of epidemiologic importance because the infection is usually field-contracted. Here the important tick vectors are considered to be *Rhipicephalus evertsi* and possibly other tick species associated with their rodent hosts.

In 1921, Megaw described a spotted-fever-like illness occurring in India

which he believed to result from a tick bite. Later, cases were discussed by Topping, Heilig & Naidu (170) and specific complement-fixation tests with serums of three of these from Mysore, India, indicated that the disease was closely related to the spotted fever group. Philip and co-workers (127) reviewed recent field and laboratory findings and reported on studies of a strain of rickettsia isolated from *R. sanguineus* ticks caught on the premises of a former tick-typhus patient in Kashmir, northern India. Early results of their studies indicate that the rickettsia is a member of the Rocky Mountain spotted fever group and is closely related to fièvre boutonneuse.

North Queensland tick typhus.—Andrew, Bonnin & Williams (1) recently observed in Australian troops a rickettsial disease which produces a syndrome resembling fièvre boutonneuse and tentatively named it North Queensland tick typhus. On circumstantial evidence of tick bite in seven cases (two with eschar at the site) the disease was presumed to be transmitted by ticks, *Ixodes holocyclus*. Since experimental transmission by other ixodids has not been accomplished, proof of actual tick transmission of this infection requires more critical evidence (121). A Weil-Felix reaction was positive for *Proteus* OX19 and OX2 in all 12 cases observed. Rickettsias isolated in the laboratory from two of the patients were shown by Funder & Jackson (53) to give positive serological reactions with serums from patients with the disease. Growth of the etiologic agent within the nuclei of cells in tissue culture (53, 133) and results of specific serological studies by Lackman & Parker (83) indicate that the disease is related to Rocky Mountain spotted fever. Work of the latter authors suggests that the disease is not identical with any of the known members of the spotted fever group, but represents a new strain within the group, for which the name *Rickettsia australis* was proposed (121). Streeten *et al.* (168) recently reported three cases of presumed tick typhus which occurred in south Queensland. No rickettsias were isolated nor were specific serologic tests made. However, presence of an eschar, history of tick bite, and clinical syndrome suggested that these were cases of the same rickettsiosis.

Siberian tick typhus.—The Russian literature on this disease has been reviewed by Crocker *et al.* (39). It is a typhus-like disease transmitted by ticks and occurring in central Asia, central Siberia, and the maritime provinces of the Far East. The illness is mild and characterized by the occurrence of a lesion at the site of tick bite, headache, and rash. Vectors in the Far East are believed to be *Haemaphysalis concinna* and *Dermacentor silvarum*, while in central Siberia *Dermacentor nuttallii* is involved. Natural infection with the rickettsia and its transovarial transmission have been demonstrated in these three tick species. The natural occurrence of the disease is broadly diversified, since these ticks attack a wide variety of large and small animals, and infection has been found in various rodent hosts of the ticks.

The relationship of Siberian tick typhus to other rickettsial diseases is at present uncertain. Recently, Crocker *et al.* (39) reported a study of four

rickettsial strains, which were supposedly the etiologic agents of this disease, and showed all four to be indistinguishable from *R. prowazekii*. However, it is doubtful that these four strains represent the actual etiologic agent of the disease described as Siberian tick typhus, since they were labelled otherwise when received and were not transmissible by *D. andersoni* (121).

Rickettsialpox.—Rickettsialpox is the name given by Huebner, Stamps, & Armstrong (66) to a rickettsial disease first discovered and described in New York City [see (36)]. Recent studies of the clinical manifestations of the disease have been reported (6, 147). It is a mild typhus-like illness, characterized by an initial lesion, fever, and a varicelliform rash. The vector was shown (63) to be a mite, *Allodermanyssus sanguineus*, parasitic on house mice which invaded the premises of patients. Experimental transmission by the more ubiquitous tropical rat mite, *Bdellonyssus bacoti*, indicates it should not be overlooked as a potential vector in other foci (126a).

The physical and biological characteristics of the etiologic agent, named *Rickettsia akari*, were summarized by Huebner (63). The organism grows profusely in the yolk sac of chicken eggs from which serologic antigens can be prepared, and ether extraction of such cultures yields a soluble antigen as do *R. prowazekii* and *R. rickettsii*. Immunological studies indicate that *R. akari* is related to but not identical with other rickettsias of the spotted fever group.

Since its discovery in New York City, the disease has been recognized in Massachusetts and Connecticut. Two clinically and serologically proved cases in Boston have been described (47), and *R. akari* has been recovered from house mice trapped on the premises (51). Report of eight cases in Connecticut has just come in (175a) with characteristic clinical and epidemiological circumstances and rising complement-fixing titers which indicate probability of a new focus. Presence of the disease in French Equatorial Africa, as reported by LeGac & Giroud (84), should be confirmed by isolation and characterization of the causative rickettsia.

Present evidence indicates that the house mouse, *Mus musculus*, is the natural animal reservoir from which mites transmit the disease to man. The causative agent has been isolated from man, mites, and house mice, all inhabiting the same premises. The presence and appearance of an initial lesion, together with epidemiologic and laboratory evidence, suggests that transmission of rickettsialpox to man is through the bite of infected mites.

The differential clinical diagnosis is considered by Barker (6) and others, but final diagnosis of the disease must rely on laboratory tests, as is the case in most other typhus-like diseases. The complement-fixation test with yolk-sac antigens of *R. akari* is a useful aid in confirming the clinical diagnosis (6, 147) and in identifying the agent recovered from patients or other sources (51, 66). However, a considerable degree of cross reaction has been observed with the rickettsialpox and spotted fever antigens, though the higher titers have usually occurred with the homologous antigen. In many cases, however,

rickettsialpox antisera and spotted fever antisera have each shown higher titers with the heterologous rather than with the homologous antigen, regardless of whether soluble or washed rickettsial body antigens have been used (37, 147). Thus, the complement-fixation test alone does not differentiate conclusively between spotted fever and rickettsialpox. These observations indicate the importance of isolating the causative agent and determining its biologic and other characteristics when attempting to establish the occurrence of rickettsialpox in areas where the disease previously has not been observed. The Weil-Felix reactions with *Proteus* OX antigens were not considered significant in this disease by investigators, but recently Rose found titers to *Proteus* OX19 ranging from 1:160 to 1:640 in 9 of 80 cases studied (148a). Among these there was usually a concomitant rise in titer against OX2, but not against OXK antigens. These patients had not received rickettsial vaccine previously, nor was there any indication of *Proteus* infection in them.

Good results in the isolation of *R. akari* from patients, house mice, or mites have been obtained by use of laboratory mice. In some cases, however, detection of infection in these animals following primary inoculation must rely on blind passages. This point is emphasized by the work of Fuller *et al.* (51), from which it is evident also that the liver and spleen of house mice are more satisfactory than brain tissue in attempted isolation of the agent from this animal.

Other Rickettsioses

Tsutsugamushi disease.—Considerable expansion of knowledge concerning the epidemiology of this chigger mite-borne disease in primitive areas in the Asiatic-Pacific region occurred as a result of World War II. Summary articles of this experience have beeen published (98, 122), and the startling contrast in this expansive military operation is made between almost 7500 cases of this disease in the American military forces alone compared to only 64 total cases of epidemic typhus, a spectacular historic fact considering the grim toll exacted by the latter in campaigns of the past. Differences in effective vector control must have been important here though it is difficult to evaluate the part played by vaccination against epidemic typhus (38).

At present the disease is known in southern and eastern Asia, northern Australia, certain islands adjoining these regions, and a number of islands in Malaysia and Melanesia. These foci are encompassed within a rough geographic quadrangle with Japan and the New Hebrides Islands at the eastern angles, and western India and the Chagos Achipelago (Diego Garcia) in the west. For more detailed information of the geographic distribution of the disease see Philip (120). More recent reported foci within this region include the Shantung (73) and Kansu [see (3)] provinces of China, and an area on the slopes of Mount Fuji, Japan, where a possible new mite vector may be involved (82). There is still no definite evidence for presence of this disease

in Africa though reports have implied its presence chiefly on serologic grounds [see (50)].

The epidemiology of scrub typhus is, for the most part, a reflection of the local ecology of the two principal mite vectors, *Trombicula akamushi* and *Trombicula deliensis*, and their major rat hosts. Kohls and co-workers in New Guinea furnished the first conclusive proof of natural infection in *T. deliensis* (under its synonymic name, *T. walchi*) which was later confirmed by others in Burma and India [see (128)] and in Malaya (129, 174).

Other mite species may be involved either in transmission of the disease to man, or in the intermurid maintenance of natural infections (128). This is strongly supported by the important discovery of Traub, Frick & Diercks (174) of natural infection in the trombiculid mite, *Euschöngastia indica*, collected from tree squirrels in Malaya. Ability of this species to transmit the infection in nature remains to be proved, but it is not known to bite man. Kalra [see (3)], in India, recovered a strain from a pool of mites which appeared to consist of only *Schöngastia ligula*. Exclusion of the presence of any *T. deliensis* mites in such injected pools must be assured for reliance on positive results.

There are several recent important contributions to rearing techniques of trombiculid mites (72, 80) which should enhance the experimental approach to several problems of the natural cycle of infection. Incidental to mite vector studies in Malaya, Traub & Frick (173) suggest that, though infection of larval *T. akamushi* and *T. deliensis* can be transovarially acquired, it may require "reactivation" similar to the phenomenon of spotted fever in ticks. They were unsuccessful in demonstrating *Rickettsia tsutsugamushi* in unfed mites of all stages in hyperendemic areas by injection, though feeding of larvae from adults from the same locations produced infection.

The most important vertebrate hosts of the vector mites are various kinds of native ground-frequenting rats of the genus *Rattus* in the more tropical areas and mice in Japan. The zoological range of naturally infected hosts includes rodents and insectivores [see (128)]. Although isolation of infection has been made repeatedly from mites parasitizing various of the vertebrate hosts, including rodents, marsupials, and insectivores, Philip, Traub & Smadel (129) reported the first avian source of infected mites from quail in Malaya. The observation emphasizes the suggested role (3, 126) of wandering birds in the epidemiology of scrub typhus. (Circulating infection was also demonstrated for varying periods up to 26 days in some experimental fowl.) In addition, the quail were found to be more heavily infested with vector mites than were rats in the same area, as many as 600 mites being found on a single bird. Audy & Harrison (3) report some 18,500 larvae of *T. akamushi* recovered from nine quail collected in Malaya.

Humidity is an important factor in the ecology of the vector mites, their activity on and in the soil being largely dependent upon moisture. This is reflected in the pronounced seasonal incidence in the population of larval *T.*

deliensis with consequent effect upon the incidence of human infections discernible, at least, in those endemic areas exhibiting marked seasonal changes. Appearance of these mites during rainy periods was found to be correlated with the incidence of scrub typhus in lower and central Burma (3), and in India (81). In Malaya, where no seasonal incidence in the disease has been noted in the past, there are noticeable decreases in larval mite population accompanying dry periods (3, 129, 173). Moreover, Traub & Frick (173) noted that cases occurred near Kuala Lumpur during the wet season but not during the dry periods. The effect of moisture on the activity of vector larval mites was emphasized by the observation (129) that the period of greatest diurnal activity in attacking volunteers was in the morning while the dew was evaporating.

Audy (2) and Audy & Harrison (3) discuss the ecology of the vectors and their animal hosts as well as the relation of scrub typhus to terrain and season. From the occurrence of the disease in exposed military populations during the war it became apparent that scrub typhus was not strictly related to any special vegetation but was encountered more often in certain ecologic types of terrain that promote native rat harborages and vector mite activity. There appears to be no certain evidence of infection of man within a deep primary jungle. However, the presence of endemic foci in such areas has been suggested from cases which appeared to be contracted in dense forest [see (122)] and from surveys which have shown that jungle rats may harbor numerous *T. deliensis* mites. More recently Traub, Frick & Diercks (174) furnished factual evidence of infected jungle foci demonstrating natural infection in three species of jungle rats and in mites obtained from jungle tree squirrels.

Though usually observed in rural environments, the disease has been reported in patients residing in Calcutta, Bombay, and in other urban areas [see (122, 165)]. Recently, Gispen, Smit & Westerman (57) described an etiologically proved epidemic of scrub typhus in Batavia, Java, in which rural sources of infection could be excluded. The urban source appeared to be a restricted area overgrown with alang grass and situated along a canal in the Duku district. A rat and mite survey of several districts of Batavia by Gispen (55) revealed that rats infested with akamushi mites were found only in the district of Duku, the epidemic area, and were caught only in or near the houses situated along the border of the grassy area along the canal (which is reminiscent of the "back yard" infections in the Pescadores Islands).

Q fever.—A rickettsial disease of animals transmissible to man, Q fever is rapidly being recognized as an important public health problem. It has been identified in Australia, North America, Panama, Western Europe, the Middle East, Africa, and possibly China [see (28, 109)]. The disease departs rather radically from the classical, typhus-like rickettsioses. The lack of the usual rash and Weil-Felix serology, the small part that arthropods

play in human infection, together with unique differences in certain biological and physical properties of the etiologic agent have warranted differentiation of *C. burnetii* on a generic level (124).

The maintenance in nature of Q fever involves arthropods (ticks) and probably certain of the native wild animals which they parasitize. However, unlike other rickettsioses, this disease appears capable of a separate maintenance, independent of an arthropod vector, by means of a chain of successive infections in certain of the larger domestic animals, viz., cattle, sheep, and goats. To what extent this may occur also among susceptible wild animals is unknown. Infected domestic animals of the above species constitute one reservoir of the disease and have been shown to be the immediate source of most human infections. There is no proved instance of tick transmission of Q fever to man, although a possible instance is recorded (43). Neither is there any definite evidence for a relationship between the rickettsial parasitic cycle in nature and in domestic animals.

Several recent publications contain excellent reviews and adequate bibliographies on the general subject and history of Q fever (41, 97, 115). Others review its epidemiologic and epizootiologic aspects covering studies carried on in California during the past few years (64, 85, 86). While studies in this area have provided the most complete data available on the epidemiology and epizootiology of Q fever occurring endemically, it should be remembered that the natural history of this disease may differ according to various environments and local human customs.

Ticks are the only animal ectoparasites known to be naturally infected with *C. burnetii*, and the agent has been shown to be adapted to transovarial transmission in them; recently human body lice were reported to be infected in Africa [see (54)]. The rickettsia has been isolated from at least 17 species of ticks found in North America, Australia, Africa (16, 17, 54, 115, 118), and Western Europe (46, 116, 119). Additional species are demonstrated experimental vectors, while in still others *C. burnetii* persists but cannot be transmitted by biting (115). Uniformly negative results have been obtained in attempts to isolate the rickettsia from field collections of mites and of several species of insects including flies, mosquitoes, fleas, and animal lice (29, 32, 65, 115). Mosquitoes failed to transmit infection either mechanically or after incubation, but tick feces may be rich in viable organisms for over a year and a half. This inoculum infected guinea pigs through the unbroken skin as well as by inhalation (123). The importance of tick sources in Australian abattoir infections, however, needs further study in view of recent findings elsewhere.

Natural infection of wild animals with *C. burnetii* has been demonstrated only in a marsupial bandicoot in Australia and in gerbils in Africa [see (115)]. However, since many species of small mammals and birds are susceptible experimentally (115), it seems likely that natural infection will be found in additional species. Isolation of this rickettsia from domestic animals is

reported for cattle, sheep, and goats only. The presence of complement-fixing antibodies in dogs (29) suggests a natural infection in them, particularly since they are known to be susceptible experimentally (115). Serological tests on other domestic animals such as horses, swine, and poultry have been negative (87).

In southern California, an early discovery revealed that milk from a large number of dairy herds was infected wih *C. burnetii* [see (64)], and present information indicates that the disease is maintained readily in such herds in the Los Angeles milkshed area. The most important factors accounting for its maintenance there appear to be the constant introduction of susceptible animals into the herds and the conditions which insure a continuous source of infection for them. In the Los Angeles area approximately 60,000 dairy cows are imported annually to replace unprofitable cattle (8), and more than 40 per cent of such imported cattle may acquire Q fever within six months after arrival on infected premises (64, 93). Ample opportunity for exposure is provided as the environment in infected dairies is constantly contaminated with *C. burnetii* present in milk and placentas of infected cattle (65, 92). These facts, together with the known ability of the agent to survive under various unfavorable conditions (124, 140), insure a continuously contaminated environment.

The common mode of transmission and route of infection in dairy cattle are unknown. Certain observations suggested the disease to be a localized infection of the mammary gland, and the route of infection to be through the teat canal during the milking process (70, 167). Experimental tests of this hypothesis by Stoenner (167) indicated that cow-to-cow spread by this means is unlikely. The method of air-borne spread, also considered, gains support from the demonstrated susceptibility of cows to infection via the respiratory tract (167) and the presence of *C. burnetii* in dust-laden air of premises harboring dairy cattle (40).

The disease is established in the sheep and goat populations of certain regions. In northern California, serologic surveys showed a high prevalence of infection in individual herds of these animals (87) probably spread by the air-borne route from animal sources (71, 178), since both species are susceptible to intranasal infection (25).

The major factor in the spread of Q fever to man apparently is associated with infected livestock, but the epidemiologic pattern of the disease may vary from one geographical area to another. This fact was made clear particularly by the extensive epidemiologic studies on the disease occurring in southern (8, 12), and in northern California (31, 88). An instance of infection of shiphands during exportation of goats (30) emphasizes a manner in which recipient areas may become contaminated.

In southern California the most frequent and important sources of human infections were shown to be local dairy cattle and their raw products, particularly milk. The majority of infections in this area were found to oc-

cur in persons exposed to dairy cattle because of residence near a dairy or livestock yards, occupation in the dairy or livestock industries, or through household use of raw milk. Ingestion may not be the only route, if at all, by which infections are acquired by persons exposed only through use of raw milk. Thus, in a large group of creamery workers who had no exposure to livestock, it was found that a history of close occupational contact with raw milk was more closely related to Q fever infections than was a history of household use of this product (64). There was no apparent variation in seasonal incidence of human cases in southern California, and the infections occurred predominantly in occupational males, aged 20 to 59 years.

In northern California, on the other hand, evidence was accumulated which indicated that sheep and goats were more frequently implicated in the epidemiology of the infection than were cattle. The majority of 350 cases studied there occurred in individuals having contact with cattle, sheep, or goats, by virtue of either occupation or residence. Household use of raw milk did not appear to be an important epidemiological factor in this area. In further contrast to observations made in the southern part of the state, the northern cases exhibited a definite seasonal variation in incidence; 70 per cent of the 350 cases occurred during the months of March, April, and May. This seasonal incidence in northern California coincides with the lambing season when greatest contamination of the environment probably occurs through milk and placentas, while in southern California calving is not seasonal and dairy herds produce milk the year around.

The specific modes of transmission of Q fever from livestock to man have not been clearly defined. A hypothesis made by Clark and co-workers (31, 32) to account for 20 to 30 per cent of California cases not directly related to livestock was that contaminated clothing of persons who had been in direct contact with infected livestock or their secretions or excreta might serve to carry the infection to persons seldom or never exposed directly to these animals. This thesis is supported by several reported instances in which isolated cases of Q fever have occurred under conditions strongly suggesting that the infections were acquired through handling contaminated clothing, straw, wool, or other materials which had been transported from some infected premises (10, 152, 177). Regardless of the manner of exposure, transfer of the infection to man is considered to be most likely through the medium of contaminated air, either by droplet infection or by dust [see (31, 41)]. Epidemiologic studies of many civilian, military, and laboratory outbreaks of Q fever have suggested this mode of transmission, and experiments on volunteers (18) indicate that man is susceptible to infection via the respiratory route. The sources of environmental contamination and presence of *C. burnetii* in the atmosphere of these foci already have been mentioned.

Trench fever.—The rickettsial nature of this disease has been variously discussed and questioned since its recognition in troops in Europe during World War I (169). The role of the body louse in transmitting the disease in

man was established. A rickettsial agent said to grow extracellularly in the guts of lice fed on trench fever cases was described as *Rickettsia quintana* Schmincke (syn. *Rickettsia wolhynica* Jungmann and Kuczynski). On the other hand, a morphologically similar extracellular rickettsia was found in lice fed on presumably healthy individuals and appeared to be nonpathogenic for man.

Since 1942, many cases have been reported among troops in eastern Europe, and more recent laboratory studies of the disease have been made [see review by Kostrzewski (79)]. These investigations have confirmed and extended many of the earlier findings but do not appear to have made any important advances concerning the biology of the etiological agent. Because of the unreliability of a clinical diagnosis, lack of a suitable serologic test, or of a susceptible laboratory animal, evidence of the identity of the causative agent of trench fever has been afforded only by means of infection of human volunteers and lice.

Confirming the findings of earlier workers, Mooser *et al.* (103) and Weyer (179) recently reported that all attempts to transmit the disease to the usual laboratory animals, including the intranasal inoculation of mice, were negative; also *R. quintana* could not be cultured in fertile hens' eggs. However, of considerable importance, if confirmed, is Codeleoncini's (33) report of the susceptibility of baboons to infection with trench fever.

The rickettsial agglutination test with rickettsias from infected lice, although of theoretic importance, has been considered impractical particularly because of variations in purity, sensitivity, and specificity of different preparations [see (11, 179)]. However, Kostrzewski (79) was able to prepare satisfactory serologic antigens from dried infected louse guts, though only in limited quantity. The Weil-Felix reaction in trench fever has been considered negative or doubtful (33, 107). Recently, Beiling & Oelrichs (11) observed that an intracutaneous injection of suspensions of living *R. quintana* from lice produced skin lesions in rabbits, which were, moreover, inhibited by human trench fever convalescent serum. Mooser *et al.* (103) were unable to recover rickettsias from such intracutaneous lesions by louse inoculation. These investigators and others have shown the value for diagnosis of suspected cases of trench fever in the demonstration of extracellular rickettsias in clean laboratory-bred lice after feeding on patients.

Some of the more recent studies have an interesting bearing upon the question of the rickettsial nature of trench fever. Patients with mild and inapparent cases, as well as with relapses of trench fever, have been observed, and rickettsias have been demonstrated in lice fed on such patients (79, 102). Moreover, relapses of the disease have occurred as late as one to three years after the original infection, and Kostrzewski (79) reports demonstration of *R. quintana* in the blood four, five, and even eight years after infection. These facts probably explain the reported recovery in lice of extracellular rickettsias from presumably disease-free individuals. In addition, Mooser

et al. (103) pointed out that it is sometimes difficult to infect persons with material from lice known to be infected with the agent of trench fever. This may explain, in part, other earlier reported failures to infect volunteers with so-called nonpathogenic strains recovered from supposedly healthy persons, further emphasized in Kostrzewski's (79) excellent discussion on the epidemiology of trench fever observed among persons employed in feeding lice for Weigl's epidemic-type vaccine.

Three epidemics among people similarly employed were reported by Mosing (107) and by Herzig (60) in Poland, and by Codeleoncini (33) in Ethiopia. Studies of these outbreaks provided strong evidence for their rickettsial etiology. Furthermore, Codeleoncini (33) and Mooser *et al.* (103) believed that these epidemics were outbreaks of trench fever and were caused by a single rickettsial species, *R. quintana*. In recent transmission tests by Mooser *et al.* (103), lice fed on patients with trench fever were shown to acquire rickettsias, the disease was produced in volunteers with material from these lice, and rickettsias were again shown to develop in fresh lice fed on these infected volunteers. It would appear from evidence contained in the above studies and other evidence that the beliefs of Mooser and his colleagues (103) are correct, and that *R. quintana* is an organism of potentially varying pathogenicity, and that *Rickettsia pediculi* Munk & da Rocha Lima 1917 and *Rickettsia weiglii* Mosing 1936 are probably synonyms of *R. quintana* Scmincke 1917.

IMMUNIZATION

Tsutsugamushi disease.—Immunization of man against scrub typhus has been attempted with both inactivated organisms and live attenuated strains. Inactivated vaccines capable of protecting mice against the disease have been prepared from the lungs or spleens of rodents and from agar tissue cultures of the rickettsias, and more recently from infected chick-embryo yolk sacs (4). Despite the promising laboratory results, these vaccines have not proved of value in protecting man against infection acquired under conditions of field exposure (13, 26, 154, 159).

Vaccination of volunteers against the disease with a living vaccine was carried out by Smadel *et al.* (157). In these tests a subclinical infection was induced by inoculation of an attenuated strain (Gilliam) of *R. tsutsugamushi* and the resulting clinical symptoms suppressed by a chemoprophylactic regimen with chloramphenicol. The authors discussed the indirect evidence which suggests that these vaccinated volunteers were immune, although their resistance to reinoculation with the rickettsia was not determined. Previous studies by these workers had shown the necessity of reinstituting treatment because of the unexpected occurrence of relapses (154).

The existence of differences in the antigenic structure of strains of *R. tsutsugamushi* [see (122)] probably affects the usefulness of living vaccines as well as of inactivated ones. Studies of Smadel *et al.* (156) and of Kawamura

et al. (76) indicate that immunity to the homologous strain of infecting *R. tsutsugamushi* probably lasts for at least several years, but that resistance to heterologous strains lasts for only a few months. Smadel and co-workers inoculated with living rickettsias a number of persons who had recovered from proved scrub typhus acquired at some previous time and found them to be solidly resistant to reinfection with the homologous strain of *R. tsutsugamushi* for one to two months, and in one instance for three and one half years. On the other hand, 11 of 16 individuals infected with a known or presumed heterologous strain one to two years after the original disease, possessed little, if any, demonstrable immunity. Kawamura and associates infected volunteers with the reported attenuated Pescadores strain and found them immune to the heterologous, virulent Niigata strain when the reinoculation was made only eight days after defervescence of the original disease. In this connection, it may be pointed out that Philip (122) found different members of the same Japanese family on a Niigata farm to have antibodies against a New Guinea strain (Karp) from an infection contracted in one year and against a Burma strain (Gilliam) in the year following.

Preparation of the lyophilized living vaccine used in the studies just discussed was described by Jackson & Smadel (69). Following the demonstration by Bovarnick, Miller & Snyder (21) of the favorable effect of buffered solutions containing sucrose, or other substances, on the survival of rickettsias, Jackson & Smadel (69) tested similar suspending menstrua for their value in preserving the viability of dried *R. tsutsugamushi*. They found solutions containing 0.2 *M* sucrose plus appropriate buffer salts to be superior to skim milk, and to yield suspensions of lyophilized *R. tsutsugamushi* in which the infectious end points were stable during storage at 5° C. or −20° C.

Q fever.—The accelerated interest in Q fever has led to studies on specific prophylaxis against this disease. The vaccines prepared during the early work on Q fever [see (99)], although capable of exhibiting a protective effect in experimental animals, had not been evaluated in man [but see Cox (38)]. More recently, ether-extracted yolk-sac vaccines prepared by the method employed for typhus vaccines have been tested in man as well as in guinea pigs. Smadel and co-workers (158) found these vaccines to be capable of protecting guinea pigs against lethal doses of *C. burnetii* and of stimulating the development of complement-fixing antibodies in man, but they obtained no information on the resistance of these vaccinated persons to infection with Q fever. Meiklejohn & Lennette (99) investigated the protective effect of similar vaccines in laboratory personnel exposed to infection. Vaccination was followed in most persons by the appearance of complement-fixing antibodies, but the titers fell considerably within four to five months afterwards. Booster injections were given at about three-month intervals to keep the antibody levels elevated. Lennette (85) notes that no cases of frank clinical Q fever have occurred among nearly 100 vaccinated personnel who were exposed for varying periods of time during two years of intensive Q fever in-

vestigations, both in the laboratory and in the field. Cox (38) has also reviewed evidence that a polyvalent vaccine (spotted fever plus Q fever) prepared from tick tissues probably protected laboratory personnel. In view of the high incidence of clinically manifest infections among workers in other laboratories engaged in Q fever studies, such results indicate that immunization with these vaccines is a valuable aid in attempts to protect laboratory personnel who are continuously exposed to infection.

A preliminary trial, in southern California, of ether-extracted yolk-sac vaccines by Luoto, Winn & Huebner (93) indicates that these vaccines may be effectively used to reduce infection rates among dairy cattle under the environmental conditions of the study.

TERRAMYCIN THERAPY

Several articles appearing in 1947 and 1948 reviewed the developments made in the chemotherapy of rickettsial infections to that time (44, 161, 171), and a later review by Eaton (42) included reference to the antirickettsial activity of the newer antibiotics, chloramphenicol and aureomycin. The dramatic success of these in treatment (and prophylaxis) of the rickettsioses is memorialized by a statement in the Jubilee Volume of the Institute for Medical Research in Malaya regarding the conquest of scrub typhus "Overnight as it were, a once severe and often mortal disease, centuries old, much feared by planter and serving soldier alike, had become trivial" (43a).

Since these reviews, terramycin, another *Streptomyces*-derived antibiotic has been reported (45). This was shown to have antirickettsial activity when tested against a wide variety of rickettsial infections in chick embryos (7, 114, 148, 155, 163), and in mice (148, 155, 163), and to have distinct therapeutic value in the treatment of patients with scrub typhus (5, 155), epidemic typhus (77), murine typhus (7), rickettsialpox (148), and Rocky Mountain spotted fever (7, 136, 150).

In comparative tests, terramycin was found to be more active, by weight, than aureomycin or chloramphenicol against rickettsial infections in chick embryos (68, 114, 148, 155, 163). This was true for all of the several rickettsial species tested. The superiority of terramycin over the other antibiotics was particularly evident in tests against *C. burnetii* in chick embryos. While this rickettsia was found to be relatively insusceptible to chloromycetin, it was essentially as susceptible to small doses of terramycin as were *R. tsutsugamushi* and *R. rickettsii* (68, 155). The drug appears to be of definite value for the treatment of Q fever infections of man. Guinchi (58) reported results of treatment of 10 patients selected because of the severity of their infections. Terramycin was given orally, in daily doses of 2 to 3 gm., with a total averaging 14 gm. over a 5-day period. The data indicate that the treatment usually was begun toward the latter part of the first or early part of the second week of the illness. The author summarized the fairly uniform response in all patients as follows: a sudden temperature drop between the

24th and 36th hour after inauguration of treatment, general improvement in symptoms, and a rapid regression of the radiological signs. The uniform results obtained, particularly in the rapid disappearance of fever, indicate that terramycin may be highly effective in the treatment of Q fever in man. Further studies employing a larger group of patients should be made.

In the treatment of scrub typhus patients, Bailey *et al.* (5) found that in certain instances terramycin produced as prompt control of the disease as did aureomycin or chloramphenicol, but that in other cases the responses were slower in appearing.

Powell *et al.* (136) reported favorable results in treatment of seven cases of Rocky Mountain spotted fever with terramycin. The average duration of fever after beginning the antibiotic was 60 hr., which compares favorably with the results obtained with aureomycin and chloramphenicol. Similarly favorable results in the treatment of spotted fever with terramycin in Brazil were reported by Magalhães *et al.* (95).

Killough & Magill (77) treated five patients with epidemic typhus in Cairo, Egypt, with terramycin and obtained a uniformly rapid response. Four of the patients became afebrile within three days, while the fifth, moribund on admission, became afebrile by the sixth day. Subjective and objective improvement occurred within 24 hr. of the beginning of treatment in all five patients.

LITERATURE CITED

1. Andrew, R., Bonnin, J. M., and Williams, S., *Med. J. Australia*, **33,** 253–58 (1946)
2. Audy, J. R., *Bull. Inst. Med. Research, Federated Malay States*, No. 1, pp. 1–82 (1949)
3. Audy, J. R., and Harrison, J. L., *Trans. Roy. Soc. Trop. Med. Hyg.*, **44,** 371–95 (1951)
4. Bailey, C. A., Diercks, F. H., and Proffitt, J. E., *J. Immunol.*, **60,** 431–41 (1948)
5. Bailey, C. A., Ley, H. L., Jr., Diercks, F. H., Lewthwaite, R., and Smadel, J. E., *Antibiotics and Chemotherapy*, **1,** 16–30 (1951)
6. Barker, L. P., *J. Am. Med. Assoc.*, **141,** 1119–23 (1949)
7. Bauer, R. E., Parker, R. T., Hall, H. E., Benson, J. F., Joslin, B. S., Hightower, J. A., Snyder, M. J., Venable, S. J., and Woodward, T. E., *Ann. N. Y. Acad. Sci.*, **53,** 395–406 (1950)
8. Beck, M. D., Bell, J. A., Shaw, E. W., and Huebner, R. J., *U. S. Pub. Health Service, Pub. Health Repts.*, **64,** 41–56 (1949)
9. Beck, M. D., and Van Allen, A., *Am. J. Hyg.*, **45,** 335–54 (1947)
10. Beeman, E. A., *U. S. Pub. Health Service, Pub. Health Repts.*, **65,** 88–92 (1950)
11. Beiling, R., and Oelrichs, L., *Z. Hyg. Infektionskrankh.*, **127,** 41–48 (1947)
12. Bell, J. A., Beck, M. D., and Huebner, R. J., *J. Am. Med. Assoc.*, **142,** 868–72 (1950)
13. Berge, T. O., Gauld, R. L., and Kitaoka, M., *Am. J. Hyg.*, **50,** 337–42 (1949)
14. Blanc, G., and Baltazard, M., *Bull. soc. path. exotique*, **33,** 25–32 (1940)
15. Blanc, G., and Baltazard, M., *Arch. inst. Pasteur Maroc.*, **2,** 535–715 (1944),
16. Blanc, G., and Bruneau, J., *Bull. acad. natl. méd.*, **133,** 115–17 (1949)
17. Blanc, G., Bruneau, J., and Chabaud, A., *Compt. rend.*, **230,** 474–75 (1950)
18. Blanc, G., Bruneau, J., Poitrot, R., Delage, B., *Bull. acad. natl. méd.*, **132,** 243–50 (1948)
19. Bovarnick, M. R., and Miller, J. C., *J. Biol. Chem.*, **184,** 661–76 (1950)
20. Bovarnick, M. R., and Snyder, J. C., *J. Exptl. Med.*, **89,** 561–65 (1949)
21. Bovarnick, M. R., Miller, J. C., and Snyder, J. C., *J. Bact.*, **59,** 509–22 (1950)
22. Brigham, G. D., and Bengtson, I. A., *U. S. Pub. Health Service, Pub. Health Repts.*, **60,** 29–46 (1945)
23. Bustamante, M. E., and Varela, G., *Rev. inst. salubridad y enfermedad. trop. (Mex.)*, **8,** 139–41 (1947)
24. Callot, J., and Vendrely, R., *Compt. rend. soc. biol.*, **142,** 396–97 (1948)
25. Caminopetros, J., *Proc. 4th Intern. Congr. Trop. Med. Malaria*, 441–49 (1948)
26. Card, W. I., and Walker, J. M., *Lancet*, **I,** 481–83 (1947)
27. Castañeda, M. R., *Proc. 4th Intern. Congr. Trop. Med. Malaria*, 408–13 (1948)
28. Chang, N. C., Zia, S. H., Liu, F. T., and Chao, S. H., *Chinese Med. J.*, **69,** 35–45 (1951)
29. Clark, W. H., Bogucki, A. S., Lennette, E. H., Dean, B. H., and Walker, J. R., *Am. J. Hyg.*, **54,** 15–24 (1951)
30. Clark, W. H., Lennette, E. H., and Romer, M. S., *Am. J. Hyg.*, **54,** 35–43 (1951)
31. Clark, W. H., Lennette, E. H., and Romer, M. S., *Am. J. Hyg.*, **54,** 319–30 (1951)
32. Clark, W. H., Romer, M. S., Holmes, M. A., Welsh, H. H., Lennette, E. H., and Abinanti, F. R., *Am. J. Hyg.*, **54,** 25–34 (1951)
33. Codeleoncini, E., *Boll. ital. med. igiene Trop.*, **6,** 129–51 (1946)
34. Cohen, S. S., *J. Immunol.*, **65,** 475–83 (1950)

35. Cohen, S. S., and Chargaff, E., *J. Biol. Chem.*, **154,** 691–704 (1944)
36. Cox, H. R., in *Viral and Rickettsial Infections of Man*, 493–515 (Rivers, T. M., Ed., J. B. Lippincott Co., Philadelphia, Pa., 587 pp., 1948).
37. Cox, H. R., *Diagnosis of Viral and Rickettsial Infections*, 133–46 (Columbia Univ. Press, New York, 153 pp., 1949)
38. Cox, H. R., *Arch. ges. Virusforsch.*, **4,** 518–33 (1951)
39. Crocker, T. T., Bennett, B. L., Jackson, E. B., Snyder, M. J., Samdel, J. E., Gauld, R. L., and Gordon, M. K., *U. S. Pub. Health Service, Pub. Health Repts.*, **65,** 383–94 (1950)
40. DeLay, P. D., Lennette, E. H., and DeOme, K. B., *J. Immunol.*, **65,** 211–20 (1950)
41. Dyer, R. E., *Am. J. Pub. Health*, **49,** 471–77 (1949)
42. Eaton, M. D., *Ann. Rev. Microbiol.*, **4,** 223–46 (1950)
43. Eklund, C. M., Parker, R. R., and Lackman, D. B., *U. S. Pub. Health Service, Pub. Health Repts.*, **62,** 1413–16 (1947)
43a. Field, J. W., *Studies Inst. Med. Research Federation Malaya*, No. 25, 37–57 (1951)
44. Findlay, G. M., *Trop. Diseases Bull.*, **45,** 553–64 (1948)
45. Finlay, A. C., Hobby, G. L., Pán, S. Y., Regna, P., Routien, J. B., Seeley, D. B., Shull, G. M., Sobin, B. A., Solomons, I. A., Vinson, J. W., and Kane, J. H., *Science*, **111,** 85 (1950)
46. Fonseca, F., Pinto, M. R., Colaco, A. F., Oliveira, J., Branco, F., Da Gama, M. M., Franco, J. S., and Lacerda, M. T., *Clin. Contemporanea*, **5,** 1–23 (1951)
47. Franklin, J. F., Wasserman, E., and Fuller, H. S., *New Engl. J. Med.*, **244,** 509–11 (1951)
48. Freeman, G., Castillo Sologuren, F., and Espinosa, H., *Am. J. Trop. Med.*, **29,** 71–78 (1949)
49. Freeman, G., Varela, G., Plotz, H., and Ortiz Mariotte, C., *Am. J. Trop. Med.*, **29,** 63–69 (1949)
50. Freyche, M. J., and Deutschman, Z., *World Health Org. Epidem. Vital. Stat. Rept.*, **3,** 161–201 (1950)
51. Fuller, H. S., Murray, E. S., Ayers, J. C., Snyder, J. C., and Potash, L., *Am. J. Hyg.*, **54,** 82–100 (1951)
52. Fuller, H. S., Murray, E. S., and Snyder, J. C., *U. S. Pub. Health Service, Pub. Health Repts.*, **64,** 1287–92 (1949)
53. Funder, J. F., and Jackson, A. V., *Med. J. Australia*, **33,** 258–63 (1946)
54. Giroud, P., *Bull. World Health Org.*, **4,** 535–46 (1951)
55. Gispen, R., *Documenta Néerland. Indonesica Morbis Trop.*, **2,** 23–35 (1950)
56. Gispen, R., *Documenta Néerland Indonesica Morbis Trop.*, **2,** 225–30 (1950)
57. Gispen, R., Smit, A. M., and Westerman, C. D., *Documenta Néerland. Indonesica Morbis Trop.*, **1,** 134–141 (1949)
58. Giunchi, G., *Minerva med.*, **42,** 829–33 (1951)
59. Hawksley, J. C., and Stokes, E. J., *Lancet*, **II,** 97–99 (1950)
60. Herzig, A., *Zentr. Bakt. Parasitenk. Orig.*, **143,** 299–302 (1939)
61. Hill, E. L., and Ingraham, S. C., *U. S. Pub. Health Service, Pub. Health Repts.*, **62,** 875–81 (1947)
62. Hill, E. L., and Morlan, H. B., *U. S. Pub. Health Service, Pub. Health Repts.*, **63,** 1635–53 (1948)
63. Huebner, R. J., *The Rickettsial Diseases of Man*, 113–17 (American Association for the Advancement of Science, Washington, D. C., 247 pp., 1948)

64. Huebner, R. J., and Bell, J. A., *J. Am. Med. Assoc.*, **145,** 301–5 (1951)
65. Huebner, R. J., Jellison, W. L., Beck, M. D., Parker, R. R., and Shepard, C. C., *U. S. Pub. Health Service, Pub. Health Repts.*, **63,** 214–22 (1948)
66. Huebner, R. J., Stamps, P., and Armstrong, C., *U. S. Pub. Health Service, Pub. Health Repts.*, **61,** 1605–14 (1946)
67. Ito, H., Kumano, T., Masu, S., and Shimada, Y., *Kitasato Arch. Exptl. Med.*, **22,** 125–33 (1949)
68. Jackson, E. B., *Antibiotics and Chemotherapy*, **1,** 231–41 (1951)
69. Jackson, E. B., and Smadel, J. E., *Am. J. Hyg.*, **53,** 326–31 (1951)
70. Jellison, W. L., Ormsbee, R., Beck, M. D., Huebner, R. J., Parker, R. R., and Bell, E. J., *U. S. Pub. Health Service, Pub. Health Repts.*, **63,** 1611–18 (1948)
71. Jellison, W. L., Welsh, H. H., Elson, B. E., and Huebner, R. J., *U. S. Pub. Health Service, Pub. Heath Repts.*, **65,** 395–99 (1950)
72. Jenkins, D. W., *Ann. Entomol. Soc., Am.*, **40,** 56–68 (1946)
73. Jones, H. L., and King, J. A., *U. S. Naval Med. Bull.*, **49,** 724–26 (1949)
74. Jude, A., and Brumpt, L. C., *Bull. soc. path. exotique*, **40,** 3–8 (1947)
75. Kalra, S. L., and Rao, K. N. A., *Indian J. Med. Research*, **37,** 373–84 (1949)
76. Kawamura, R., Ito, T., Nakamura, R., Kamimura, T., and Sato, J., *Kitasota Arch. Exptl. Med.*, **14,** 75–98 (1937)
77. Killough, J. H., and Magill, G. B., *J. Am. Med. Assoc.*, **147,** 1737–40 (1951)
78. Kohls, G. M., *The Rickettsial Diseases of Man*, 83–96 (American Association for the Advancement of Science, Washington, D. C., 247 pp., 1948)
79. Kostrzewski, J., *Bull. intern. acad. polon. sci. Classe Méd.*, No. 7/10, 233–63 (1949)
80. Krishnan, K. V., Smith, R. O. A., Bose, P. N., Neogy, K. N., Roy, B. K. G., and Ghosh, M., *Indian Med. Gaz.*, **84,** 39–41 (1949)
81. Krishnan, K. V., Smith, R. O. A., Bose, P. N., Neogy, K. N., Roy, B. K. G., and Ghosh, M., *Indian Med. Gaz.*, **84,** 63–68 (1949)
82. Kuwata, T., Berge, T. O., and Philip, C. B., *J. Parasitol.*, **36,** 80–83 (1950)
83. Lackman, D. B., and Parker, R. R., *U. S. Pub. Health Service, Pub. Health Repts.*, **63,** 1624–28 (1948)
84. LeGac, P., and Giroud, P., *Bull. soc. path. exotique*, **44,** 413–15 (1951)
85. Lennette, E. H., *Bact. Revs.*, **14,** 249–58 (1950)
86. Lennette, E. H., and Clark, W. H., *J. Am. Med. Assoc.*, **145,** 306–9 (1951)
97. Lennette, E. H., Clark, W. H., and Dean, B. H., *Am. J. Trop. Med.*, **29,** 527–41 (1949)
88. Lennette, E. H., Dean, B. H., Abinanti, F. R., Clark, W. H., Winn, J. F., and Holmes, M. A., *Am. J. Hyg.*, **54,** 1–14 (1951)
89. Liu, W. T., *Chinese Med. J.*, **62,** 119–39 (1944)
90. Liu, W. T., *Am. J. Hyg.*, **45,** 58–66 (1947)
91. Loomis, W. F., *Science*, **111,** 474 (1950)
92. Luoto, L., and Huebner, R. J., *U. S. Pub. Health Service, Pub. Health Repts.*, **65,** 541–44 (1950)
93. Luoto, L., Winn, J. F., and Huebner, R. J. (Unpublished data)
94. Magalhães, O. de, *Mem. inst. Oswaldo Cruz*, **47,** 249–71 (1949)
95. Magalhães, O. de, Café, B., and Rocha, A., *Brasil-med.*, **64,** 311 (1950)
96. Manso Sato, A. E., and Pletneff, P., *Mission Regional Path. Studies Argentina*, **21,** 5–22 (1950)
97. Marmion, B. P., *J. Roy. Sanit. Inst.*, **71,** 97–102 (1951)
98. Maxcy, K. F., *The Rickettsial Diseases of Man*, 36–46 (American Association for the Advancement of Science, Washington, D. C., 247 pp., 1948)

99. Meiklejohn, G., and Lennette, E. H., *Am. J. Hyg.*, **52,** 54–64 (1950)
100. Mooser, H., *Schweiz. Med. Wochschr.*, **76,** 877–82 (1946)
101. Mooser, H., Castañeda, M. R., and Zinsser, H., *J. Exptl. Med.*, **54,** 567–75 (1931)
102. Mooser, H., Marti, H. R., and Leeman, A., *Schweiz. Z. Path. u. Bakt.*, **12,** 476–83 (1949)
103. Mooser, H., Leeman, A., Chao, S. H., and Gubler, H. U., *Schweiz. Z. Path. u. Bakt.*, **11,** 513–22 (1948)
104. Morgan, H. R., Neva, F. A., Fahey, R. J., and Finland, M., *New Engl. J. Med.*, **238,** 871–73 (1948)
105. Morlan, H. B., Hill, E. L., and Schubert, J. H., *U. S. Pub. Health Service, Pub. Health Repts.*, **65,** 57–63 (1950)
106. Morlan, H. B., and Hines, V. D., *U. S. Pub. Health Service, Pub. Health Repts.*, **66,** 1052–57 (1951)
107. Mosing, H., *Arch. inst. Pasteur Tunis*, **25,** 373–87 (1936)
108. Murray, E. S., Baehr, G., Shwartzman, G., Mandelbaum, R. A., Rosenthal, N., Doane, J. C., Weiss, L. B., Cohen, S., Snyder, J. C., *J. Am. Med. Assoc.*, **142,** 1059–66 (1950)
109. Murray, E. S., Djakovic, P., Ljupsa, F., and Snyder, J. C., *U. S. Pub. Health Service, Pub. Health Repts.*, **66,** 1032–37 (1951)
110. Murray, E. S., Psorn, T., Djakovic, P., Sielski, S., Broz, V., Ljupsa, F., Gaon, J., Pavlevíc, R., and Snyder, J. C., *Am. J. Pub. Health*, **41,** 1359–69 (1951)
111. Murray, E. S., and Snyder, J. C., *Am. J. Hyg.*, **53,** 22–32 (1951)
112. Ormsbee, R. A., *J. Bact.*, **63,** 73–86 (1952)
112a. Ormsbee, R. A. (Unpublished data)
113. Ormsbee, R. A., Lackman, D. B., and Pickens, E. G., *J. Immunol.*, **67,** 257–63 (1951)
114. Ormsbee, R. A., and Pickens, E. G., *J. Immunol.*, **67,** 437–48 (1951)
115. Parker, R. R., Bell, E. J., and Stoenner, H. G., *J. Am. Vet. Med. Assoc.*, **114,** 55–60; 124–30 (1949)
116. Parker, R. R., DePrada, J., Bell, E. J., and Lackman, D. B., *U. S. Pub. Health Service, Pub. Health Repts.*, **65,** 1616–18 (1949)
117. Parker, R. R., and Oliphant, J. W., *Communicable Diseases*, 719–52 (Lea & Febiger, Philadelphia, Pa., 1035 pp., 1950)
107a. Parker, R. R., Pickens, E. G., Lackman, D. B., Bell, E. J., and Thrailkill, F. B., *U. S. Pub. Health Service, Pub. Health Repts.*, **66,** 455–63 (1951)
118. Parker, R. R., and Sussman, O., *U. S. Pub. Health Service, Pub. Health Repts.*, **64,** 1159–60 (1949)
119. Perez, G. F., Clavero, G., and Hernandez, S., *Rev. sanidad e hig. publica, Madrid*, **23,** 489–96 (1949)
120. Philip, C. B., in *Communicable Diseases*, 753–70 (Puller, R., Ed., Lea & Febiger, Philadelphia, Pa., 1035 pp., 1950)
121. Philip, C. B., in *Communicable Diseases*, 781–88 (Pullen, R., Ed., Lee & Febiger, Philadelphia, Pa., 1035 pp., 1950)
122. Philip, C. B., *J. Parasitol.*, **34,** 169–91 (1948)
123. Philip, C. B., *J. Parasitol.*, **34,** 457–64 (1948)
124. Philip, C. B., *U. S. Pub. Health Service, Pub. Health Repts.*, **63,** 58 (1948)
125. Philip, C. B., in *The Rickettsial Diseases of Man*, 97–112 (F. R. Moulton, Ed., American Association for the Advancement of Science, Washington, D. C., 247 pp., 1948)

126. Philip, C. B., *Sci. Monthly*, **69**, 281–89 (1949)
126a. Philip, C. B., and Hughes, L. E., *Am. J. Trop. Med.*, **28**, 697–705 (1948)
127. Philip, C. B., Hughes, L. E., Rao, K. N. A., and Kalra, L., *Abstracts Intern. Congr. Microbiol. 5th Congr.*, 115–16 (Rio de Janeiro, 1950)
128. Philip, C. B., and Kohls, G. M., *Proc. 4th Intern. Congr. Trop. Med. Malaria*, 1656–63 (1948)
129. Philip, C. B., Traub, R., and Smadel, J. E., *Am. J. Hyg.*, **50**, 63–74 (1949)
130. Pijper, A., and Crocker, C. G., *S. African Med. J.*, **12**, 613–30 (1938)
131. Plotz, H., *1st Inter-American Congr. on Typhus Fever, Mexico City*, 7–13 (1945)
132. Plotz, H., Reagan, R. L., and Wertman, K., *Proc. Soc. Exptl. Biol. Med.*, **55**, 173–76 (1944)
133. Plotz, H., Smadel, J. E., Bennett, B. L., Reagan, R. L., and Snyder, M. J., *Med. J. Australia*, **33**, 263–68 (1946)
134. Pollard, M., Wilson, D. J., Livesay, H. R., and Woodland, J. C., *Texas Repts. Biol. Med.*, **4**, 446–51 (1946)
135. Pomalez Lebron, A., *Puerto Rico J. Pub. Health Trop. Med.*, **23**, 393–407 (1948)
136. Powell, A. M., Snyder, M. J., Minor, J. V., Jr., Benson, J. F., and Woodward, T. E., *Bull. Johns Hopkins Hosp.*, **89**, 30–38 (1951)
137. Pshenichnov, A. W., *Trop. Diseases Bull.*, **41**, 836 (1944)
138. *U. S. Pub. Health Service, Communicable Disease Center Bull.*, 50–51 (July, August, September, 1948)
139. Rabinowitz, E., Aschner, M., and Grossowicz, N., *Proc. Soc. Exptl. Biol. Med.*, **67**, 469–70 (1948)
140. Ransom, S. E., and Huebner, R. J., *Am. J. Hyg.*, **53**, 110–19 (1951)
141. Rickard, E. R., *Am. J. Hyg.*, **53**, 207–16 (1951)
142. Rickard, E. R., *Am. J. Trop. Med.*, **31**, 306–10 (1951)
143. Rickard, E. R., and Worth, C. B., *Am. J. Hyg.*, **53**, 332–36 (1951)
144. Ris, H., and Fox, J. P., *J. Exptl. Med.*, **89**, 681–86 (1949)
145. Rodaniche, E. C de., and Rodaniche, A., *Am. J. Trop. Med.*, **30**, 511–17 (1950)
146. Rodriques, P. M., and Travassos, J., *Hosp. O. Rio de Janeiro*, **34**, 55–69 (1948)
147. Rose, H. M., *Ann. Internal Med.*, **31**, 871–83 (1949)
148. Rose, H. M., *Ann. N. Y. Acad. Sci.*, **53**, 385–94 (1950)
148a. Rose, H. M. (Personal communication)
149. Sadusk, J. F., *J. Am. Med. Assoc.*, **133**, 1192–99 (1947)
150. Schoenbach, E. B., Bryer, M. S., and Long, P. H., *Ann. N. Y. Acad. Sci.*, **53**, 245–52 (1950)
151. Shepard, C. C., and Topping, N. H., *J. Immunol.*, **55**, 97–102 (1947)
152. Sigel, M. M., Scott, T. F. M., Henle, W., and Janton, O. H., *Am. J. Pub. Health*, **40**, 524–32 (1950)
153. Silva Goytia, R., *Medicina, México*, **32**, 1–7 (1952)
154. Smadel, J. E., Bailey, C. A., and Diercks, F. H., *Am. J. Hyg.*, **51**, 229–41 (1950)
155. Smadel, J. E., Jackson, E. B., and Ley, H. L., Jr., *Ann. N. Y. Acad. Sci.*, **53**, 375–84 (1950)
156. Smadel, J. E., Ley, H. L., Jr., Diercks, F. H., and Traub, R., *Arch. Path.*, **50**, 847–61 (1950)
157. Smadel, J. E., Ley, H. L., Jr., Diercks, F. H., Traub, R., Tipton, J., and Frick, L. P., *Am. J. Hyg.*, **53**, 317–25 (1951)
158. Smadel, J. E., Snyder, M. J., and Robbins, F. C., *Am. J. Hyg.*, **47**, 71–81 (1948)
159. Smadel, J. E., Traub, R., Ley, H. L., Jr., Philip, C. B., Woodward, T. E., and Lewthwaite, R., *Am. J. Hyg.*, **50**, 75–91 (1949)

160. Smith, J. D., and Stoker, M. G. P., *Brit. J. Exptl. Path.*, **32,** 433–41 (1951)
161. Snyder, J. C., in *The Rickettsial Diseases of Man*, 169, 77 (F. R. Moulton, Ed., American Association Advancement of Science, Washington, D. C., 247 pp., 1948)
162. Snyder, J. C., and Davis, B. D., *Federation Proc.*, **10,** 419 (1951)
163. Snyder, J. C., Fagan, R., Wells, E. B., Wick, H. C., and Miller, J. C., *Ann. N. Y. Acad. Sci.*, **53,** 362–74 (1950)
164. Snyder, J. C., and Wheeler, C. M., *J. Exptl. Med.*, **82,** 1–20 (1945)
165. Soman, D. W., and Das Menon, V. K., *Indian Med. Gaz.*, **83,** 17–20 (1948)
166. Starsyk, J., *Przeglad Epidemiol.*, **2,** 257–59 (1948); *Trop. Diseases Bull.*, **47,** 222 (1950)
167. Stoenner, H. G., *J. Am. Vet. Med. Assoc.*, **118,** 170–74 (1951)
168. Streeten, G. E. W., Cohen, R. S., Gutteridge, N. M., Wilmer N. B., Brown, H. E., Smith, D. J. W., and Derrick, E. H., *Med. J. Australia*, **35,** 372–73 (1948)
169. Strong, R. P., *Stitt's Diagnosis, Prevention and Treatment of Tropical Diseases*, 984–96 (The Blakiston Co., Philadelphia, Pa., 1747 pp., 1944)
170. Topping, N. H., Heilig, R., and Naidu, V. R., *U. S. Pub. Health Service, Pub. Health Repts.*, **58,** 1208–10 (1943)
171. Topping, N. H., and Shepard, C. C., *Ann. Rev. Microbiol.*, **1,** 333–50 (1947)
172. Tovarnickij, V. I., Krontovskaja, M. K., and Ceburkina, N. V., *Nature*, **158,** 912 (946)
173. Traub, R., and Frick, L. P., *Am. J. Hyg.*, **51,** 242–47 (1950)
174. Traub, R., Frick, L. P., and Diercks, F. H., *Am. J. Hyg.*, **51,** 269–73 (1950)
175. Travassos, J., *Proc. 4th Intern. Congr. Trop. Med. Malaria*, 414–21 (1948)
175a. U. S. Public Health Service, *Comm. Disease Sum.* (Feb. 2, 1952)
176. Van Meter, J. C., and Oleson, J. J., *Science*, **113,** 273 (1951)
177. Wegmann, T., *Schweiz. med. Wochschr.*, **78,** 529–31 (1948)
178. Welsh, H. H., Lennette, E. H., Abinanti, F. R., and Winn, J. F., *U. S. Pub. Health Service, Pub. Health Repts.*, **66,** 1473–77 (1951)
179. Weyer, F., *Zentr. Bakt. Parasitenk., Orig.*, **152,** 403–14 (1948)
180. Wisseman, C. L., Jr., Hahn, F. E., Jackson, E. B., and Smadel, J. E., *Federation Proc.*, **10,** 424 (1951)
181. Wisseman, C. L., Jr., Jackson, E. B., Hahn, F. E., Ley, A. C., and Smadel, J. E., *J. Immunol.*, **67,** 123–36 (1951)
182. Worms, R., *Bull. mém. soc. méd. hôp., Paris*, **66,** 54–57 (1950)
183. Worth, C. B., and Rickard, E. R., *Am. J. Trop. Med.*, **31,** 295–98 (1951)
184. Worth, C. B., and Rickard, E. R., *Am. J. Trop. Med.*, **31,** 301–5 (1951)
185. Zinsser, H., *Am. J. Hyg.*, **20,** 513–32 (1934)

CLASSIFICATION AND NOMENCLATURE OF VIRUSES[1]

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"Solomon, we are told, knew the names of all the spirits, and, having their names, he held them subject to his will. The parable may be applied to bacteriology, as to many other sciences." The quotation is from an essay on "The Nomenclature and Classification of Micro-organisms" written just over twenty years ago by the reviewer's father. Later in the essay we read "Judgement must be suspended . . . in the case of the invisible viruses or so-called 'filter-passing' organisms. Here our ignorance is almost complete: they are possibly a heterogeneous group, but in the case of creatures which we cannot see and whose very existence is in many cases a matter of inference only, it is idle to talk of classification in the usual sense" [Andrewes (5)].

At that time, no one would have disputed that conclusion; but in the intervening years we have learned a very great deal about the "so-called filter-passing organisms," and many feel that the time is now ripe to name and to classify them.

One may name and classify in several ways. There are trivial names: mumps, tobacco mosaic, polyeder-krankheit. Such names are perhaps the best to use when we recognise a disease and some of the properties of its causative agent, but know almost nothing of its relationships to other viruses. Again, one can use letters and numbers as is done in referring to the types of Coxsackie virus. Finally there is the Linnean binomial system with names for species, genera, families and other groups. Use of this system is traditionally linked with classification, for in its essence it tries to denote phylogenetic relationships, close ones between the species of a genus, less close with those of other genera, relations of a different order between the several genera of a family, and so forth. Thus, as soon as we attempt a nomenclature of this type, we are involved in classification, and as soon as we attempt a rational, orderly classification we have to consider very carefully before breaking away from the Linnean species-genus system. It is true that there are other systems of nomenclature, as we shall see when considering plant viruses. Here, and still more in such attempts as there are to classify animal viruses, there is a tendency to classify the diseases and not the viruses which cause them. Such a course is a tempting one to follow because the diseases are easy to observe and the intrinsic properties of the viruses difficult to discover. It is, however, obvious that we could never do this with the bacteria, or we should group together all the causes of tonsillitis and find the diphtheria bacillus, haemolytic streptococci, and the organisms of Vincent's angina all in one genus. There is no more justification for applying such a method in the virus field.

[1] The survey of the literature pertaining to this review was concluded in February, 1952.

How great is the need for action in this field? Animal and plant virologists will return very different answers. The viruses attacking animals are relatively fewer in numbers; some, such as that of variola, are very well known; the trivial names given by the discoverers of new ones are universally used; those who work with viruses in the laboratory are accustomed to an international scientific literature; hence, the confusion caused by lack of an orderly system is minimal. As knowledge advances, the situation is, to be sure, likely to alter. The plant virologists on the other hand, are already much embarrassed by a profusion of names given to one and the same virus. Many plant viruses attack numerous and often unrelated species of plants and produce different symptoms in different species, so that no system based either on host preferences or on symptomatology yields a logical result. Nevertheless, plant pathologists are agreed on the existence of chaos which they would like to see replaced by order, and upon the desirability of agreement as to a single system of nomenclature. That is unfortunately the extent of their agreement, for most of them seem to believe very firmly in one or other of several entirely incompatible solutions.

Considerations which affect plant pathologists but do not seem to bother students of animal viruses are these: are the conceptions of genus and species rightly applicable to viruses at all? Are they living things? Are they not chemicals, ultimately to be classified on chemical lines? Have we any right to assume that the ancestors of a virus were necessarily other viruses and that they are not derived separately from cell constituents of their hosts? If so, the Linnean system which carries the implication of genetic relationship would clearly be inappropriate. These are questions which may still be the subject of discussion many years hence. The reviewer feels that they may well be safely neglected at present, since viruses as they now exist behave in so many ways like autonomous organisms.

The official nomenclature of animals and plants is under the control of international zoological and botanical nomenclature committees, which have codes laying down rules and recommendations. There is now a similar body for microbiological nomenclature, and its code has been published (Buchanan, St. John Brooks & Breed (16)). The Fourth International Microbiological Congress held in Copenhagen in 1947 decided that viruses came within the field of jurisdiction of the microbiological code. The botanists, however, have taken an interest in the nomenclature of plant viruses and their International Congress in 1930 appointed an International Committee on plant virus classification and nomenclature. It adopted a tentative system of nomenclature not of the Linnean type [Johnson (40)]. Further work on this was held up because of the war, and in the meantime a comprehensive system of classification on Linnean lines was published by Holmes (37, 38). At the 1951 International Botanical Congress at Stockholm a proposal was made to hand the nomenclature of plant viruses over to the microbiologists, but this was not accepted. It would seem essential that some agreement should be reached on this matter. If botanists and

microbiologists sponsor different systems of nomenclature, confusion will become worse confounded. To the reviewer it would seem absurd to have the classification and nomenclature of animal and plant viruses under two entirely different bodies.

PLANT VIRUSES

Numerical Systems

A brief account now follows of the various systems which have been proposed for dealing with the plant viruses. It will be clear that all of them are based mainly on host specificity and symptomatology. The first comprehensive system was that of Johnson (39). He proposed that the virus' name should consist of that of the host on which it was first found (either English or Latin names being used) followed by a number indicating the chronological order of discovery of the virus attacking that host. Thus the tobacco mosaic virus would be Tobacco virus 1. This system was essentially the one tentatively adopted by the botanists in 1930. Kenneth Smith's (61) system is also in principle the same but uses the generic Latin names of the plant; thus tobacco mosaic becomes Nicotiana virus 1. Letters are used for strains derived from a typical strain. Either of these systems has the disadvantage that it requires the memorising of numbers and that "new" viruses must continually have numbers allotted to them by some international authority. On the other hand, such a system has the advantage that it is so clearly a temporary expedient that nobody can mistakenly believe it to be intended as a permanent scientific system of nomenclature, linked with classification or based on natural relationships.

Binomial Systems

Fawcett (29) proposed to form the generic names of viruses from the generic name of the first described host, adding the suffix "vir": thus tobacco mosaic becomes *Nicotianaevir commune*. Specific names are purely descriptive. This is in a sense a hybrid between numerical and a more orthodox binomial system. It has some of the advantages of each; thus the suffix "vir" at once informs us that we are dealing with something rather different from ordinary Linnean binomials.

Holmes' (37) system was proposed in 1939 and modified in 1948 (38) when it was incorporated in a supplement to the Sixth Edition of *Bergey's Manual of Determinative Bacteriology*. He considered that the decision of the Microbiological Congress in 1947 to include viruses under its code of nomenclature gave him the right to propose if he chose an extension of the Linnean system to all viruses. (In 1939 he had dealt only with plant viruses.) The system is ostensibly based on natural relationships among viruses but actually the emphasis is preponderantly on host reactions and symptoms. The viruses are placed in an order Virales (14): in 1939 he had given them the higher rank of a Kingdom, the Vira, of equal status with animals and plants.

There are three suborders: Phagineae, infecting bacteria; Phytophagineae, infecting higher plants; and Zoophagineae, infecting animals. The Phytophagineae in turn are subdivided into sixteen genera, grouped in six families on the basis of differences in symptoms produced and in methods of transmission.

McKinney's system (49) accorded the plant viruses the rank of a division of the plant kingdom, Viriphyta and was on similar lines to Holmes', being based on transmission and host reactions. Eighteen genera were erected and divided between two families.

Thornberry (66) suggested an order Biovirales containing three families, the Rickettsiaceae, Phytoviraceae and Zooviraceae. Bacteriophages were included as one of the genera of the Phytoviraceae. All the viruses affecting higher plants were included in one genus *Phytovirus*. Specific names were a compound of the host name and a noteworthy symptom; thus tobacco mosaic now became *Phytovirus nicomosaicum* Thornberry.

Valleau (67, 68) only dealt with the viruses affecting tobacco, and modified Holmes' names. He thought that one genus such as *Marmor* should be used as a temporary dumping-ground for all imperfectly understood viruses (Vira imperfecta). He called tobacco mosaic *Musivum tabaci* Valleau.

In 1949 a Committee of the American Phytopathological Society (22) sent out a questionnaire to 190 American workers, asking whether they preferred a numerical or a binomial system: if the former, whether they liked better that of Johnson or of Smith; if the latter, whether they voted for that of Fawcett, Holmes, McKinney, Thornberry or Valleau. One hundred twenty-six workers replied; of these, 84 voted for a binomial, 41 for a numerical system and three abstained. For the separate systems the votes were: Johnson 24, Smith 17, Fawcett 4, Holmes 66, McKinney 6, Thornberry 1, Valleau 4, others 6. Thus about two to one preferred a binomial system to a numerical one and a majority of these preferred Holmes' system to any of its rivals. Two comments may be made concerning this poll. It did not give sufficient opportunity for the voicing of opinion by people who like a binomial system but think all existing proposals for such to be thoroughly unsound. Second, as will appear later, workers in other countries are not much attracted by any of the binomial systems yet put forward, and any permanent system needs to be acceptable on an international basis.

Bawden (8) has criticized on several occasions attempts to classify on a basis of host affinities and host reactions. He writes, "It would seem as reasonable to expect to classify flowering plants by the reaction to a specific virus as it is to classify viruses by the symptoms they produce in a given number of hosts." He quotes from the introduction to Bergey's Manual, "No organism can be classified before we have determined its morphological, cultural, physiological and pathogenic characters," and continues, "it is worth noting that pathogenic characters come last. It is odd that similar considerations are not considered to apply equally to viruses. . . . " Turning from destructive to constructive criticism, he suggests that serological

affinities may prove valuable. Plant viruses so far studied in this way may be put into seventeen serological groups. He admits that "immunological tests are limited in scope to the grouping of closely related strains around a type species." Another method is the detection of mutual antagonism or interference as evidence of relationship between two viruses, but this cannot be applied where the viruses to be compared do not share a common host. The method is not without pitfalls in the animal virus field where two otherwise unrelated viruses such as those of influenza and equine encephalomyelitis may show mutual interference when growing in eggs. Possibly interference may at times indicate only competition for similar metabolites in the host cell.

Methods of transmission may be helpful, for vectors of individual viruses are usually confined to nearly related species of insects. Thus viruses transmitted by aphids are not commonly transmitted also by leafhoppers or thrips. Existence or not of a latent period before the vector becomes infectious is a matter depending apparently on the properties of the virus rather than on those of the vector [Watson & Roberts (72)].

Bawden feels that grouping into genera and families presents the greatest difficulties, but finally concludes that one can begin to see certain groups as emerging into some sort of definition from the confusion. Thus "the many strains of tobacco mosaic and of potato virus X can be grouped together: a second group might contain potato virus Y, henbane mosaic, tobacco etch and soyabean mosaic viruses, all of which have rod-shaped particles and share many properties. . . . A third could contain tomato bushy stunt, tobacco necrosis, southern bean mosaic, turnip yellow mosaic and squash mosaic viruses, all of which crystallise, have spherical particles, similar gross constitutions and seem to inactivate in many similar ways." Other apparently natural groups are those causing "yellows" (here common symptom-production is linked with other similarities), strains of tomato spotted wilt virus, and viruses related to tobacco ringspot. Finally "grouping should be based on fundamental characters of the virus particles themselves . . . " and therefore "many viruses will remain unclassifiable for a long time." Bawden approves of relegating these for the time to a limbo of Vira imperfecta analogous to that of Fungi imperfecti reserved for difficult, unclassifiable fungi.

A British committee, a subcommittee on plant virus classification of the plant pests and diseases committee (65), considered this matter and came to similar conclusions as to the unwisdom of basing classification mainly on symptoms and host preferences. They favoured the use, pending a decision by an International congress, of a "list of common names of virus diseases," such as that published in 1946 by the Commonwealth Mycological Institute (23); this would clearly have to be revised at intervals. The object was apparently to avoid confusion in naming diseases while leaving the field open for further developments in naming and classifying viruses.

Opinion in Holland seems to be on similar lines. Limasset (45) in France,

on the other hand, favours a modification of Holmes' system and writes of "Holmes a qui revient le merite d'avoir appliqué la nomenclature linnéenne aux virus, rendant ainsi possible l'établissement progressif d'une classification naturelle."

ANIMAL VIRUSES

While the plant virologist is bewildered by a multiplicity of systems, his colleague in animal pathology is faced with an entirely different situation. He has not felt either a dire need for an orderly nomenclature nor the existence of a factual basis on which such a thing could be set up. Many writers of textbooks or general reviews on animal viruses have roughly classified their material, usually on a basis of what tissues or organs were attacked, but there has been no suggestion in these proposals that the viruses themselves were being naturally grouped. Tissue affinities were first felt important by Borrel (13) who in 1903 wrote of Epithelioses. Levaditi (42) paid particular attention to the group which he called Les Ectodermoses neurotropes, including diseases caused by viruses having affinity for ectoderm, either of the skin, cornea, or central nervous system. Later he [Levaditi & Voet (43)] subdivided this group according to their preferential attack on different tissues of ectodermal origin. In 1937 Levaditi and Lepine (44) classified viruses into eight groups: (*a*) Ectodermoses (foot-and-mouth disease, vesicular stomatitis, ectromelia, vaccinia); (*b*) Ecto-endodermoses (canary-pox, laryngo-tracheitis); (*c*) Mesodermo-endodermoses (fowl plague and Newcastle disease); (*d*) Ectodermoses neurotropes (a number of purely neurotropic virus infections); (*e*) Endodermoses neurotropes (yellow fever); (*f*) Mesodermoses (lymphogranuloma venereum); (*g*) Septicémies (influenza, Rift Valley fever); (*h*) Ultravirus neoformatif (Rous sarcoma). Such a classification has not found general favour. Neither has anything developed from Goodpasture's (33) attempt to separate "cytotropic viruses," having very close relationships with cells, from other "filterable viruses." Ledingham & Barratt (41) pointed out that ectodermoses such as vaccinia included attack on mesodermal cells. Much other knowledge acquired in intervening years has shown how variable and unreliable are tissue affinities as guides to classification.

Verge & Goret (69) made attempts at classifying on similar lines the viruses of veterinary interest. Their primary grouping was into "naturally pathogenic viruses" and "saprophytic viruses, experimentally pathogenic." The first group was subdivided into cultivable (bovine pleuropneumonia and agalactia contagiosium) and cytotropic. About seventy viruses in the last group were divided into:

(*A*) Ectotropes
- (1) Cytocinétiques
- (2) Cytolytiques
  - (*a*) Neurotropes
  - (*b*) Dermatropes
  - (*c*) Dermo-neurotropes

(3) Mixtes
(*B*) Endotropes
(1) à affinites pour l'appareil respiratoire
(2) à affinites pour l'appareil digestif
(*C*) Mesotropes
(*D*) Bacteriotropes (phages)

As to nomenclature, no comprehensive attempts were made before Holmes (38), though Goodpasture (32) proposed the generic name *Borreliota* for the pox-group and specific names within that genus. Also Paillot (53) had suggested *Borrelina* as a generic name for some insect-pathogenic viruses.

Otherwise only sporadic suggestions for binomials for individual viruses had been made, particularly in the era when inclusion bodies were first discovered and thought to be akin to protozoa. It is true that Moshkovsky (51) and others had proposed binomials for the group of large viruses related to psittacosis. Rake (55) and others have suggested that this group should be removed from the true viruses, a matter we shall return to later.

Otherwise there was in 1948 an almost clear field for Holmes' attempt to create both a comprehensive classification of and a binomial nomenclature for the animal pathogenic viruses. Holmes' attempt has, however, such grave defects that experienced virologists have, almost with one accord, rejected it. A very few writers have been using his binomials. The large majority feel, rather naturally, that any such effort in the field of animal virology should have been left to an animal virologist. Holmes' system, as the only comprehensive one in the field, will be discussed in some detail, if only to show why it has not generally found favour and in hope of clarifying one's ideas as a guide to future projects in this field.

Holmes' System

Animal viruses are considered as a suborder *Zoophagineae* of the order *Virales*, and this is divided into six families. One of these, the *Borrelinaceae* is a natural one, comprising those inducing diseases in insects as exclusive hosts. The other five families are based almost entirely on tissue tropisms and symptomatology and each of them contains viruses which in other respects form an ill-assorted motley crew. The generic names used are for the most part euphonious, reasonably short and based on classical allusions which are in many cases neat and apposite. None of them, however, is defined save by reference to a type species and by a reference in a few words to tropisms and symptoms. The families also are not defined except as "inducing diseases of the so-and-so group." The specific names, almost all newly-coined, are in most instances perfectly suitable. Thus many people will have little quarrel with Holmes' names as names, but only with the expediency of attempting a comprehensive classification at this time, with the defects of this particular attempt at classification, and with the characters used to define the species and other groups.

Holmes includes two genera, *Borrelina* attacking lepidopterous caterpillars and *Morator*, one species attacking honey-bees, in the family Borrelinaceae, with no mention of the viruses attacking the caterpillar-like larvae of the hymenopterous sawflies, nor those parasitising diptera. Steinhaus' review of the subject and criticism of Holmes' efforts will be referred to later.

The *Borreliotaceae* or pox-group includes in Holmes' scheme, five genera. The last of these, *Molitor*, includes a variety of viruses having widely dissimilar properties and almost nothing in common but a tendency to cause cell-proliferation. Thus the highly resistant wart viruses lie cheek by jowl with that of the Rous fowl sarcoma. All the fowl tumour viruses are considered as strains of one species. The very different myxoma virus also finds itself in this strange genus. Since very many virus infections depend on a balance between proliferation and destructive processes, cell-proliferation seems a diagnostic criterion of the poorest quality. In the other four genera of this family, *Borreliota*, *Briareus*, *Scelus*, and *Hostis*, we find fourteen species, some of them clearly happy amongst their friends and relations, herpes simplex, pseudorabies and B virus for instance; others, such as ectromelia, reft from its natural affinities with the poxes and placed with herpes in the genus *Scelus;* chicken-pox and measles form another ill-assorted couple. On the whole the Borreliotaceae covers most of Levaditi's ectodermoses other than the stricter neurotropes. These latter are found in the *Erronaceae*. Here the rabies virus, *Formido inexorabilis*, naturally takes a genus to itself. Another genus *Erro* includes the arthropod-borne encephalitis viruses; these lie naturally close together though perhaps they are also related to yellow fever and other viruses which are only potentially neurotropic and are put by Holmes in a different family. *Erro bornensis*, the virus of Borna disease, has properties very different from those of the others in the genus. The third genus *Legio* seems to be rather a scrap heap for neurotropic viruses not otherwise cared for. It contains poliomyelitis, lymphocytic choriomeningitis and some others whose affinities are obscure.

Next come the *Charonaceae* or viruses of the yellow fever group. Yellow fever itself and Rift Valley fever are naturally placed together in one genus, *Charon*. The key to separate them runs: (*a*) Vectors mosquitoes, *Charon evagatus* (yellow fever); (*b*) Vectors unknown, perhaps mosquitoes, *Charon vallis* (Rift Valley fever). It now appears that the generic name *Charon* is preoccupied. The second genus *Tarpeia* includes all the very different viruses attacking the respiratory tract—influenza, the common cold, dog distemper, cat distemper, fox encephalitis, laryngo-tracheitis: their heterogeneity is such that further comment is needless. The third genus *Tortor* "viruses of the hog-cholera group" contains a number of species many of which are not well understood. Others, such as fowl plague, are probably related to influenza. Panleucopenia of cats (virus *Tortor felis*) is generally believed to be the same as cat distemper, the virus of which, *Tarpeia felis*, appears in a different genus.

The family *Trifuraceae*, causing abnormalities of the blood, contains the

virus of equine infectious anaemia alongside that of fowl leucosis: the latter would by most people be placed in close association with those causing fowl tumours. Finally the *Rabulaceae* contains the viruses causing inclusions in salivary galnds of rodents in mésalliance with that of mumps.

Discussion at Rio de Janeiro

There is little wonder that animal virologists did not welcome this attempt at comprehensive classification, and the inclination of most was to ignore it. However, its inclusion in Bergey's Manual must have seemed to some to give it a stamp of authority, and it soon appeared that virologists would do well to make their attitude clear. Otherwise they might find themselves faced with a highly distasteful *fait accompli*.

The matter was therefore brought up at the Fifth International Congress of Microbiology held at Rio de Janeiro in August, 1950 [Virus Subcommittee (70), Andrewes (4)]. The reviewer was asked to convene an unofficial committee to consider the subject, and this was, at Rio, given official status under the International Nomenclature Committee. As a preliminary step, opinions were sought by correspondence from 120 well-known virologists in 22 countries; over 80 replies were received. Plant and animal virologists were in roughly equal numbers. The questionnaire elicited the following information. Plant virologists were almost exactly evenly divided as to whether they wanted a binomial nomenclature. On the whole, those from the United States favoured it; those from elsewhere were against. On the other hand, about two out of every three animal virologists believed that a binomial nomenclature should eventually be applicable in their field; only one in five, however, wanted to see the system generally applied forthwith, and the verdict was almost unanimously against acceptance of Holmes' system.

Eight members of the virus subcommittee were able to discuss the question at Rio; only one of these was a plant virologist (Dr. Bitancourt). As a result of their recommendations the International Nomenclature Committee recommended that consideration of the starting date for scientific nomenclature of viruses be deferred to the next Congress, and expressed an opinion that "until studies on viruses now in progress have advanced further, the use of any comprehensive system of scientific nomenclature for them is unwise." They further asked for the co-operation of all virologists in assisting the subcommittee.

These resolutions were unanimously passed by the Plenary Session of the Congress. The subcommittee was asked to divide into sections dealing with animal, plant and bacterial viruses respectively, these sections to come together again later. In order to implement the views expressed, the subcommittee considered and agreed upon the principles upon which the classification of at least animal viruses should be based. These were:

(*a*) Morphology and methods of reproduction
(*b*) Chemical composition and physical properties

(*c*) Immunological properties
(*d*) Susceptibility to physical and chemical agents
(*e*) Natural methods of transmission
(*f*) Host, tissue, and cell tropisms
(*g*) Pathology, including inclusion-body formation
(*h*) Symptomatology (this was purposely placed last as of minor importance).

It was felt that it would be profitable for small groups of two or three specialists to consider certain better understood groups of viruses; these could be studied and provisional names proposed in hopes of better establishing what principles could best be used. The virus groups in question were:

(*a*) Psittacosis-lymphogranuloma group (Chlamydozoaceae)
(*b*) Insect-pathogenic viruses
(*c*) Pox group
(*d*) Influenza group (influenza, mumps, fowl plague)
(*e*) Anthropod-borne encephalitides

Reports upon these viruses will presumably be considered at the next Microbiological Congress.

An attempted assessment of the position as regards some animal viruses follows. The eight "principles" just described will be found to be varyingly applicable to different viruses. It will be noted that size and resistance to chemical agents seem to be properties of particular value. Thus Andrewes and Horstmann (2) found that separation of viruses on a basis of resistance to ethyl ether grouped together viruses which were similar by other tests. Resistance to bile salts [Smith (60)] gave rather similar groupings. On the other hand, there was no correlation with precipitability by protamine [Warren *et al.* (71)]. In discussing these groups reference will be made to opinions expressed at a Conference on Virus and Rickettsial Classification and Nomenclature held under the auspices of the New York Academy of Sciences in January 1952; the results of the general discussions at this Conference will be described later.

Psittacosis-lymphogranuloma group.—Consideration of this group at once brings up one of the more controversial questions of virus taxonomy. Should this group be included with or excluded from the true viruses? Rake (55) prefers to exclude them on several grounds: they stain positively by Castañeda's method; they apparently have a containing membrane; they divide by binary fission; they are susceptible to a variety of chemotherapeutic agents such as penicillin and, at times, sulphonamides; a life cycle including plaques and elementary bodies is demonstrable [Bedson & Bland (9)]. Many of them are interrelated by complement-fixation tests. There is no doubt that they form a relatively homogeneous group but so do other groups of viruses, and many virologists do not agree with Rake's proposal.

It is clear that there is no sharp line of demarcation between bacteria and viruses. It is relatively easy to say that a given agent is a bacterium, a rickettsia, one of the psittacosis group or a typical virus, for the two debatable divisions, the rickettsiae and the Chlamydozoaceae are fairly well defined. The difficulty comes in knowing whether to put these two groups on the bacterial or viral side of the dividing line. Any such decision must be arbitrary. Their inability to grow on artificial culture media does not make them viruses, for there are bacteria which will not do this. Formation of inclusion bodies within cells is a character shared by rickettsiae and viruses. Transmission by arthropods occurs in some bacterial, many viral and most rickettsial infections, though apparently often not in Q fever.

Bacteria, as is well known, vary in sensitivity to drugs and antibiotics. Rickettsiae are, in large part, sensitive to aureomycin and chloramphenicol, but not to penicillin. Psittacosis-like virus, on the other hand, can be checked not only by these but sometimes by sulphomanides also. There are some viruses not in the psittacosis group which are sensitive to at least aureomycin and terramycin; that of atypical pneumonia of man may be one; two others are the grey lung virus of mice [Andrewes & Glover (1), Andrewes & Niven (3)] and mouse hepatitis virus [Gledhill & Andrewes (31)]. Rod-like morphology cannot readily be used as a criterion. Rickettsiae are often but not always bacillary in form; so are many of the insect pathogenic viruses, while influenza is often filamentous, and many plant viruses are also, on a smaller scale, rod-like.

Clearly the decision as to where to divide bacteria from viruses is a hard one. Many would consider a line between bacteria and rickettsiae, that is including rickettsiae with the viruses, to be as natural as any other. The taxonomy and nomenclature of the rickettsiae is, all the same, not being considered in this review, especially as the application of binomials in that field has already obtained wide acceptance [Philip (54)].

With the psittacosis group it is otherwise, and unfortunately the names which will be considered by some to hold the field according to the laws of priority are those of Brumpt (15) and Moshkovsky (51). Many of the proposed names are very long and unharmonious: *Miyagawanella lymphogranulomatis* is an example. Rake (55) has revised the family and the genera he uses seem natural ones, *Chlamydozoön* for trachoma and inclusion conjunctivitis, *Colesiota* for two other viruses causing conjunctivitis, and *Miyagawanella* for the rest. Some of Rake's species, *M. illinii* and *M. louisianae* for example, seem doubtfully entitled to specific rank, as they show only minor pathogenic and immunological differences from other species. The family, Chlamydozoaceae, is included by him as one of the order Rickettsiales, the others being Bartonellaceae and Rickettsiaceae. Some parasites of protozoa are tentatively placed in the same order.

At the New York Conference, Coles (21), Meyer (50), and Rake (57) all maintained that the Chlamydozoaceae should be grouped with the rickettsiae. Philip (54) considered that rickettsiae might be derived phylogeneti-

cally from bacterial symbionts of insects; to maintain a similar origin for Chlamydozoaceae would, perhaps, be hazardous. Both Coles and Meyer disliked the term "Chlamydozoön" (mantle animal) and the derived family name on several grounds including the criticism that the creatures were not animals, and most had no mantles. Meyer suggested that "this misnomer should be replaced by an international body of taxonomists as soon as the descriptive family features become more apparent." He suggested the generic name *Bedsonia* for the agents closely related to that of psittacosis. Others discussing this question felt doubts as to the wisdom of too hasty a decision as to the inclusion of this group with the viruses. It may prove on further study that the rickettsiae and psittacosis-like viruses divide by binary fission while other viruses do not.

Whether they are included in the viruses or not, the psittacosis group is a natural one. The morphology and life cycle of these agents are rather characteristic as are their physical properties and chemical composition so far as is known. They are all rather labile, and the pathological changes produced show similarities. On the other hand, the less important criteria—tissue and host tropisms, and symptoms produced vary rather widely.

Insect pathogenic viruses.—Steinhaus (63) published a review on the nomenclature and classification of insect viruses. He praised Holmes' initiative and considered his "classification as a provisionally satisfactory starting point" but made criticisms in matters of detail. He suggested that in future names should not be bestowed until something was known of a virus' morphology. Nevertheless, "in order to preserve continuity" he accepted "for the time being" all of Holmes' names whether the viruses in question had been seen or not. This standpoint would be a not unreasonable one if the small group of insect viruses alone was concerned, particularly as the physical properties of so many of them have been studied. If, however, Holmes' system is not adopted for viruses as a whole, the wisdom of perpetuating Holmes' names for this group only would be doubtful. Steinhaus classified the insect viruses he dealt with into four genera, adding *Paillotella* and *Bergoldia* to Holmes' two, and relying largely on the production or not of polyhedral or other types of inclusion. He criticised, however, Holmes' use in classification of the properties of the inclusion body rather than of the virus contained therein. He admitted the defects of using hosts as criteria for differentiating species, but in many instances did not know of any other applicable criterion. A key based on hosts alone would certainly seem hazardous, seeing that the range of host-specificity of insect viruses is a matter still under study. Such a system certainly led to trouble in the plant virus field. Steinhaus clearly recognised that symptomatology is a bad basis for classification and was somewhat apologetic for including "for the convenience of the reader" an account of the symptoms produced, in the descriptions of some new species. Bergold (10), discussing the question in New York, opposed the retention, even provisionally, of Holmes' names for

viruses not yet adequately characterised. He felt that the genera *Morator* (affecting honey-bee larvae) and *Paillotella* should be forgotten about, at any rate for a time. Steinhaus (64) also was prepared to consider these two as "*genera et species incertae sedis.*" Bergold further proposed the genus *Smithia* for the small spherical viruses lately described by Smith (62).

No one would, I think, deny that the insect pathogenic viruses form a natural group, particularly the better studied ones which form polyhedra. Here we have a good basis of knowledge of the virus' intrinsic properties, and we find that there is high correlation between these and biological properties. In many of the viruses of this group, for instance, a presumably latent infection is activated when the insects are bred for several generations under somewhat artificial condtions.

The pox group.—Some viruses of the pox group have been very thoroughly studied by various techniques. Electron micrography indicates that typical members are brick-shaped. Certain numbers of this group on digestion with pepsin are seen to have a central core surrounded by less opaque material; the appearances almost suggest a nucleus and cytoplasm [Dawson & Macfarlane (27)]. These viruses are all rather large and tend to be resistant to physical agents and to chemicals such as ethyl ether. Some members exhibit immunological cross-reactions. Some agglutinate fowl red cells by virtue of a virus-product rather than through the agency of intact virus particles themselves [Nagler (52)]. Most are transmitted as a result of entry through the skin, or perhaps mucous membranes, rarely and not essentially through biting insects. Many cause cell-proliferation as a preliminary to cell necrosis and most form cytoplasmic inclusion bodies; intranuclear ones are less typical. All induce skin lesions often of the "pock" type, beginning as a papule or vesicle. Most worders would probably agree that these are in general the properties which they associate with a "pox" virus. It is far more difficult than with the Chlamydozoaceae and insect pathogens to be sure of the limits of the group. Smallpox, vaccinia, infectious ectromelia, fowl-pox, swine-pox and other animal poxes form the hard core of the typical genus, *Borreliota* of Goodpasture (32). Varicella is clinically like variola, often too like it for the comfort of public health authorities: its elementary bodies are however, less brick-shaped [Ruska (58)] and its inclusions are intranuclear ones of Cowdry's (24) A-type. For many reasons it must be associated with herpes zoster and this in turn has similarities to that triad of closely related viruses, herpes simplex, pseudorabies and B virus [Burnet *et al.*, (17)]. This group of viruses may finally be placed next to the true poxes but distinct from them.

The closely related myxoma and fibroma of rabbits are of about the same size and shape as the pox-viruses and produce basically similar pathological lesions. They are, however, more labile, not being resistant to ethyl ether and may also end up in a separate but related genus.

Molluscum contagiosum forms elementary bodies rather larger than those

of the other viruses concerned. There is evidence, at present lacking for the others, that they are developed from bodies of smaller size [Rake & Black (56)]. Again this seems to be a virus belonging in the same family.

The influenza group.—On a basis of symptomatology, influenza, mumps, and fowl plague do not seem to fall together. Indeed Holmes (38) places them in three genera and mumps even in a separate family. It is their common power of agglutinating red blood cells which first suggested that they might be related [Burnet (18), Lush (47)]; and this haemagglutinating power is of course due to a common affinity for and power of enzymatic destruction of certain receptors (mucoproteins, except perhaps for influenza C). The clue seems to have been a good one, for the viruses of Influenza A, B and C, mumps, fowl plague, and Newcastle disease are of similar size and similar lability to physical and chemical agents. All grow in embryonic fluids of eggs and are found to have many tropisms in common. They show mutual interference of varying degree especially when grown in eggs but also, in some cases, in the mouse brain. Some members of the group (Influenza A, B, and C, and fowl plague) apparently exist in the form of filaments as well as spheres; it seems possible that these spheres are formed within or by the segmentation of the filaments [Chu, Dawson & Elford (20, 26)]. Newcastle disease virus also shows, by electron microscopy, elongated forms suggesting the existence of heads and tails. These appearances are, however, variable according to salt concentration and may not be analogous to the filaments of others of the group [Bang (6, 7)]. The reliability of haemagglutination as a means of characterising a particular group is naturally called in question by the discovery that quite a number of neurotropic viruses will agglutinate red cells (34). The basis of this is in most instances different from the virus-red cell reaction of influenza, but it seems advisable not to place reliance on one property of a virus to the neglect of others. The haemagglutinating neurotropic viruses are, for instance, a good deal smaller than those of the influenza group, do not show filaments and may differ also in such properties as ether-sensitivity or transmissibility by insects.

In the New York discussion, Francis (30) pointed out that Influenza C virus differed from the others in several ways, particularly in its affinity for a different type of cell-receptor. The six viruses mentioned above were generally agreed to merit consideration as a group, though possibly mumps and Newcastle disease virus would ultimately form a separate genus.

Arthropod-borne encephalitis viruses.—These form a group still harder to delimit. There is, again, a core of viruses obviously close allies: eastern, western, and Venezuelan encephalomyelitis, St. Louis and Japanese B encephalitis, West Nile Fever, and some others of the viruses isolated from mosquitoes and found to be neurotropic in mice. All are rather small, ether-sensitive, carried by mosquitoes, neurotropic, and in many instances show serological affinities. The tick-borne viruses, louping-ill and Russian spring summer encephalitis, are smaller, but probably not far removed from them. Yellow fever and Rift Valley fever may also be related viruses. These re-

semble them in many physical properties, but differ in being only potentially neurotropic, having rather an affinity for the liver, in which characteristic pathological changes may be produced. We are, however, probably safe in putting into a wholly separate category the EMC (encephalomyocarditis) group of viruses and those naturally causing encephalomyelitis in mice. These are smaller, more resistant to ether, and are more apt to be excreted in the feces than to be carried by insects. Some are clearly related to that of poliomyelitis.

The arthropod-borne encephalitis group illustrates the difficulty of reliance on antigenic makeup as a criterion. Several of these viruses show antigenic overlap, though few would dispute that they are good species. The situation differs, however, only in degree from that of Influenza A viruses, where serological sub-types are recognised; here, however, there is a fairly continuous spectrum of antigens, so that differentiation into several species is hardly practicable.

Those [Hammon (35), Enders (28), & Sabin (59)] discussing the encephlaitis group at the New York Conference were fairly well agreed as to the limits of the group, feeling that yellow fever, dengue and Rift Valley fever probably belonged with the arthropod-borne encephalitides, while the encephalomyocarditis viruses should be excluded. They felt, however, that the application of binomials to the group was premature and that for the time being the use of the current trivial names caused no great inconvenience.

Other viruses.—Of other viruses, some fall into natural groups of relatively small size; such are the fowl-tumour viruses with those of fowl leucosis and neurolymphomatosis. These are alike, so far as studied, in size, physical, and other intrinsic properties, as well as in the lesions they produce. Equally homogeneous are likely to be the various viruses causing warts of mammals.

It was suggested at the New York meeting that consideration might be given to two further, fairly well defined, groups before the 1953 Rome conference; the viruses related to poliomyelitis, and those producing intranuclear inclusions of Cowdry's type A, particularly the herpes-pseudorabies-B virus triad.

There still remains the majority of animal viruses, whose properties are so little understood that they cannot as yet be usefully placed. Measles, dog-distemper, rinderpest, and swine-fever may be related to each other, but it would at present be futile to guess. It may be significant that those we understand least are those which cannot so readily be studied in eggs or small experimental rodents. What seems clear is that while we may be able usefully to group many viruses into genera, a grouping into families and larger categories would be premature at this time.

What may prove a useful suggestion for naming animal viruses comes from Hanson (36). It is that trivial names for "new" animal viruses be devised in an orderly manner. The first name of a trinomial might indicate the apparent primary host, the second, the type of disease, the third, some other

character, immunological, geographical, etc. Thus equine encephalomyelitis (Eastern) or mouse pneumonitis (Nigg) would fall into this pattern. The system is similar to some of those proposed for plant viruses.

CLASSIFICATION OF BACTERIAL VIRUSES

Some bacteriophages, such as the well-known T series, have been studied more thoroughly than almost any other viruses, so that in a sense such agents should be particularly suitable for classification. But this requires not merely much knowledge about a few organisms, but rather a sufficiency of knowledge about many organisms and their interrelationships; that, for the phages, we do not possess. The reviewer knows of no worker in the phage field who thinks the time ripe for classifying and naming phages on the Linnean system. Holmes attempted to name and classify a few phages as such, but only a very few, and his attempt has not met with any acceptance. The principles on which bacterial viruses could be named are the same as those already discussed under viruses in general with appropriate modifications to suit the very different kind of host.

Adams, at the New York meeting, suggested using a criterion not so readily applicable to other viruses. Two unrelated phages show the phenomenon of mutual exlcusion when given access to susceptible bacteria; two related ones, however, might simultaneously infect such cells and even give evidence of mutual interchange of genes.

PLEUROPNEUMONIA-LIKE ORGANISMS

Attention has already been given to the rickettsiae as agents lying between bacteria and viruses. The pleuropneumonia-like organisms are in a somewhat similar position. At one time it was common to hear them classed as cultivable viruses, but such a view is no longer popular. The chief reason for not considering them in more detail now is doubt as to their relationships to L-forms of bacteria such as *Streptobacillus moniliformis*. This species and many others have filterable forms which may at times be propagated as such without necessarily reverting to the orthodox form from which they were derived. Such filterable or L-forms are so like the pleuropneumonia-like organisms in their morphology and life cycles that we do well to remain uncommited as to the relations of the latter either to bacteria or to viruses [Dienes (25)].

THE GENERAL DISCUSSION AT THE 1952 CONFERENCE IN NEW YORK

This conference, already incidentally referred to, was planned to permit interchange of ideas in advance of the more official discussions and decisions expected at the International Microbiological Congress in Rome in 1953. Some of the views expressed are worth quoting *verbatim*. Thus, Burnet (19) in his opening remarks supported the generic concept for viruses since "it

would be much easier if a name as expressive as *Mycobacterium* or *Clostridium* was available to cover that particular group of viruses and provoke an immediate mental picture." He would "prefer to see definitive names attached only to well-studied viruses of which a certain clone can be maintained as the type specimen of the species." He felt that action was indicated now since he did not "foresee any great spontaneous activity in fields of virology that would greatly advance the knowledge of systematic relationships." On the other hand, any classification would act as a stimulus to study of the field from an evolutionary standpoint.

Mayr, (48) speaking as a taxonomist, felt that "virologists would avoid many of the nomenclatorial difficulties of the zoologists if they would assign the authorship of their scientific names to national or international committees and charge them with the responsibility of issuing a list of official names."

Black (12) spoke of four leafhopper-borne viruses now known to multiply in their insect vectors. The existence of these served "to remind us that the separation of viruses into bacterial, plant and virus groups . . . is arbitrary and that there exist forms that bridge the gap between two of these three major classes."

Practically all speakers agreed that classification of viruses which relied too heavily on symptomatology were unnatural; also that we should proceed slowly and not attempt to classify in the absence of a reasonable modicum of knowledge. The so-called "Rio approach," the plan of first naming a few better-known groups, was approved by most. Particularly attractive to many was the notion of a catch-all group of Vira imperfecta for the viruses, especially plant viruses, which were not yet ripe for Linnean christening. This idea, originally suggested by Bawden, was based on the analogy of Fungi imperfecti, a group now containing 20,000 species in over 1000 genera. It was made necessary in that group because so many fungi were not known to exist in that phase of the life cycle on which classification was generally based. Objection was taken to the term Vira imperfecta on the grounds that incomplete virus was a term in current use in quite another connection; some such term as Vira incognita was felt to be more suitable for the catch-all group. By this plan definitive Linnean binomials would be given to viruses of which the intrinsic properties had been well studied, while a parallel system without any phylogenetic implications would be used for the Vira incognita. Some speakers, including Limasset (46), would have based the naming of these on Holmes' proposals, suitably modified. Bitancourt (11) suggested, and subsequent speakers endorsed his view, that names used for Vira incognita should have a suitable suffix, so that anyone could tell at once whether a name was a definitive one or otherwise. So far as could be judged, the idea of Vira incognita, especially if the names were suitably tagged, was felt by many to afford a useful way out of the impasse of competing systems for naming plant viruses. Its possible usefulness for the less-known animal viruses was but little discussed.

THE FUTURE OF VIRUS NOMENCLATURE

The nomenclature of plants and animals has been the subject of much controversy and change, owing largely to the fact that the earlier names were bestowed without understanding of the principles of taxonomy as we now know it, often without reference to type material, and on the basis of very inadequate descriptions. In the reviewer's opinion, such troubles could be avoided in the virus field by dating valid nomenclature in this group not from the time of Linnaeus 200 years ago, but from a date to be decided upon in the future. The questionnaire referred to earlier yielded the result that a majority (42 out of 71) of both animal and plant virologists preferred a date in the future for starting a valid nomenclature for viruses.

Very few descriptions of viruses published hitherto would satisfy those who are seriously considering the matter today. Binomials are not in common use for any viruses, and there seems therefore everything to be gained by starting with a clean sheet. If there is approval of the piecemeal method proposed at Rio, names of the groups first dealt with can be used provisionally and validated later when the official starting date is fixed. Such virus names already published as seem suitable would also be validated, but virus nomenclature need not be forever overlaid by the dead hand of bad naming, linked to descriptions which are hard to interpret and are based on unsuitable guiding princples. If, however, students of viruses take thought in time and base their classification and nomenclature on solid foundations with reference from the very beginning to type material, they can forever be free from the nightmares of change and contentiousness which bedevil nomenclature in other fields.

LITERATURE CITED

1. Andrewes, C. H., and Glover, R. E., *Brit. J. Exptl. Path.*, **26,** 379 (1945)
2. Andrewes, C. H., and Horstmann, D., *J. Gen. Microbiol.*, **3,** 290 (1949)
3. Andrewes, C. H., and Niven, J. S. F., *Brit. J. Exptl. Path.*, **31,** 767 (1950)
4. Andrewes, C. H., *Acta Path. Microbiol. Scand.*, **28,** 211 (1951)
5. Andrewes, F. W., *A System of Bacteriology*, **1,** 292 (Stationery Office, London, England, 347 pp., 1930)
6. Bang, F., *Proc. Soc. Exptl. Biol. Med.*, **63,** 5 (1946)
7. Bang, F., *Proc. Soc. Exptl. Biol. Med.*, **64,** 135 (1947)
8. Bawden, F. C., *Plant Viruses and Virus Diseases* (Chronica Botanica Co., Waltham, Mass., 272 pp., 1950)
9. Bedson, S. P., and Bland, J. O. W., *Brit. J. Exptl. Path.*, **13,** 461 (1932)
10. Bergold, G., *Ann. N. Y. Acad. Sci.* (In press)
11. Bitancourt, A., *Ann. N. Y. Acad. Sci.* (In press)
12. Black, L. M., *Ann. N. Y. Acad. Sci.* (In press)
13. Borrel, A., *Ann. inst. Pasteur*, **17,** 99 (1903)
14. Breed, R. S., Murray, E. G., and Hitchens, A. P., *J. Bact.*, **47,** 321 (1944)
15. Brumpt, E., *Ann. parasit. humaine et comparée*, **16,** 153 (1938)
16. Buchanan, R. E., St. John-Brooks, R., and Breed, R. S., *J. Bact.*, **55,** 287 (1948)

17. Burnet, F. M., Lush, D., and Jackson, A. V., *Australian J. Exptl. Biol. Med. Sci.*, **17,** 41 (1939)
18. Burnet, F. M., *Australian J. Exptl. Biol. Med. Sci.*, **20,** 81 (1942)
19. Burnet, F. M., *Ann. N. Y. Acad. Sci.* (In press)
20. Chu, C. M., Dawson, I. M., and Elford, W. J., *Lancet*, **I,** 602 (1949)
21. Coles, G. W. A., *Ann. N. Y. Acad. Sci.* (In press)
22. Committee of American Phytopathological Society on Nomenclature and Classification, *Plant Disease Reptr.*, **34,** 35 (1950)
23. Commonwealth Mycological Institute, *Rev. Applied Mycol.*, **24,** 513–56 (1946)
24. Cowdry, E. V., *Arch. Path.*, **18,** 527 (1934)
25. Dienes, L., in *Bergey's Manual of Determinative Bacteriology*, 6th ed., 1287 (Williams & Wilkins Co., Baltimore, Md., 1529 pp., 1948)
26. Dawson, I. M., and Elford, W. J., *J. Gen. Microbiol.*, **3,** 298 (1949)
27. Dawson, I. M., and Macfarlane, A. S., *Nature*, **161,** 464 (1949)
28. Enders, J., *Ann. N. Y. Acad. Sci.* (In press)
29. Fawcett, H. S., *Chronica Botan.*, **7,** 7 (1942)
30. Francis, T., *Ann. N. Y. Acad. Sci.* (In press)
31. Gledhill, A. W., and Andrewes, C. H., *Brit. J. Exptl. Path.*, **32,** 559 (1951)
32. Goodpasture, E. W., *Science*, **77,** 121 (1933)
33. Goodpasture, E. W., *Am. J. Hyg.*, **17,** 154 (1933)
34. Hallauer, C., *Handbuch der Virusforschung II Ergänzungsband*, 220 (Springer-Verlag, Vienna, Austria, 425 pp., 1950)
35. Hammon, W. M., *Ann. N. Y. Acad. Sci.* (In press)
36. Hanson, R. P., *Science*, **104,** 606 (1946)
37. Holmes, F. O., *Handbook of Phytopathogenic Viruses* (Burgess Publishing Co., Minneapolis, Minn., 221 pp., 1939)
38. Holmes, F. O., in *Bergey's Manual of Determinative Bacteriology*, 6th ed. (Williams & Wilkins Co., Baltimore, Md., 1529 pp., 1948)
39. Johnson, J., *Agr. Expt. Sta.* (*Wisc.*) *Bull.*, **76,** 1 (1927)
40. Johnson, J., *Tijdschr. Plantenziekten*, **55,** 128 (1949) (In English)
41. Ledingham, J. C. G., and Barratt, M. M., *Lancet*, **II,** 515 (1929),
42. Levaditi, C., *Les Ectodermoses Neurotropes* (Masson & Cie., Paris, France, 269 pp., 1922)
43. Levaditi, C., and Voet, J., *Compt. rend.*, **201,** 743 (1935)
44. Levaditi, C., and Lepine, P., *Les Ultravirus des Maladies Humaines* (Norbert Maloine, Paris, France, 1182 pp., 1937)
45. Limasset, P., *Ann. épiphyt.*, **14,** 283 (1948)
46. Limasset, P., *Ann. N. Y. Acad. Sci.* (In press)
47. Lush, D., *J. Comp. Path. Therap.*, **53,** 157 (1943)
48. Mayr, E., *Ann. N. Y. Acad. Sci.* (In press)
49. McKinney, H. H., *J. Wash. Acad. Sci.*, **34,** 139 (1944)
50. Meyer, K. F., *Ann. N. Y. Acad. Sci.* (In press)
51. Moshkovsky, S., *Advances in Modern Biol.* (*U.S.S.R.*), **19,** 12 (1945)
52. Nagler, F. P. O., *Australian Med. J.*, **1,** 281 (1942)
53. Paillot, A., *Compt. rend.*, **182,** 180 (1926)
54. Philip, C. B., *Am. J. Hyg.*, **37,** 301 (1943)
55. Rake, G., in *Bergey's Manual of Determinative Bacteriology*, 6th ed., 1083 (Williams & Wilkins Co., Baltimore Md., 1529 pp. 1948)
56. Rake, G., and Black, H., *J. Investigative Dermatol.*, **15,** 81 (1950)

57. Rake, G., *Ann. N. Y. Acad. Sci.* (In press)
58. Ruska, H., *Klin. Wochschr.*, **22,** 703 (1943)
59. Sabin, A. B., *Ann. N. Y. Acad. Sci.* (In press)
60. Smith, K. M., *A Text-book of Plant Virus Diseases,* v (J. & A. Churchill Ltd., London, England, vii + 615 pp., 1937)
61. Smith, K. M., *Endeavour,* **10,** 194 (1951)
62. Smith, W., *J. Path. Bact.,* **48,** 557 (1939)
63. Steinhaus, E. A., *Bact. Rev.,* **13,** 203 (1949)
64. Steinhaus, E. A., *Ann. N. Y. Acad. Sci.* (In press)
65. Subcommittee on plant virus classification of plant pests and diseases committee, *Ann. Applied Biol.,* **37,** 329 (1950)
66. Thornberry, H. H., *Phytopathology,* **31,** 23 (1941)
67. Valleau, W. D., *Chronica Botan.,* **6,** 223 (1941)
68. Valleau, W. D., *Chronica Botan.,* **7,** 152 (1942)
69. Verge, G., and Goret, P., *Rev. gén. de méd. vét.,* **44,** 261 (1935)
70. Virus subcommittee of International Commission on Bact. Nomenclature, *Intern. Bull. Bact. Nomenclature and Taxonomy,* **1** (2), 1 (1951)
71. Warren, J., Weil, M. L., Russ, S. B., and Jeffries, H., *Proc. Soc. Exptl. Biol. Med.,* **72,** 662 (1949)
72. Watson, M. A., and Roberts, F. M., *Ann. Applied Biol.,* **27,** 227 (1940)

IDENTIFICATION OF VIRUS INFECTION IN PLANT TISSUE[1]

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Introduction

Many investigators have often felt the urgent need for some quick and simple tests to identify virus infection in plant tissue. This need is most acute in research with those virus diseases whose symptoms develop slowly, sometimes requiring months or even one or more growing seasons for expression. Means of readily identifying virus-infected tissue would serve useful purposes in facilitating the establishing of virus-free stocks in nursery material, budding wood and root and tuber clones in such instances when symptom development is delayed or obscure, or especially where symptomless hosts complicate procedures. The need for a quick and simple identification of infected tissue is less pronounced in those virus diseases which can be readily transmitted mechanically to hosts that produce symptoms in a few days. Under such circumstances it is possible quickly and practicably to make test inoculations with suspected tissue.

Rapid tests designed to identify infected tissue would necessarily either identify the virus itself or some morphological or physiological host response characteristic of a given disease. While these identifications, if successful might have their greatest value in practical ways, their development would also greatly aid in an understanding of the virus and its action on the host. Unfortunately, something less than complete success has so far attended endeavors in the development of ready, simple, and reliable means of identification of virus-infected tissue. The status of information and dependability of some diganostic methods are reviewed below.

Chemical Tests

Acid tests for stone fruit viruses.—The discovery of a chemical test for phony disease of peach by Hutchins (1) in 1933 was probably the first test to be used widely. A cross section of peach root or stem is placed in a test solution composed of 1 to 5 drops of chemically pure hydrochloric acid in 25 ml. of absolute methyl alcohol. Absolute ethyl alcohol may be used, but it requires higher acidulation and heated solutions are necessary for a rapid test. After 3- to 5-min. of exposure to this solution phony peach tissue is indicated by the appearance of numerous plainly visible purplish dots distributed throughout the wood and sharply contrasting with the faintly purplish to whitish background. In contrast thin wood sections from healthy trees

[1] The survey of the literature pertaining to this review was concluded December, 1951.

are colored slightly and evenly without purplish dots. Hutchins found that root wood gave better results than stem wood and that best results were obtained if roots free of blemishes were selected. Hutchins *et al.* (2) stated that the test described above applies particularly to peach. Other *Prunus* species gave reactions that were somewhat variable, the test being excellent in some, *P. davidiana* for example, and less striking in others.

KenKnight (3, 4) has described the reaction of phony infected trees to the acid test as being somewhat analogous to the development of photographic films in that the "image" is present but invisible before treatment. The application of the acid test is likened to the developer in the photographic process.

KenKnight found that any moderately strong acid served satisfactorily for the phony test. Liquid acids such as nitric, sulfuric, hydrochloric, formic, phosphoric, phosphorous, sulfurous, lactic, acetic, and propionic proved effective without the addition of any other substance. Nitric, sulfuric, and hydrochloric acid without dilution produced almost instantaneous development at 25°C., but overdevelopment followed rapidly. When these acids were used in alcohol dilutions, the images remained readable for a period roughly twice as long as required for development. Undiluted formic, phosphoric, and phosphorous acids produced images in a matter of minutes at 25°C., but the images were not sharp and therefore somewhat unsatisfactory. Sulfurous acid required hours to produce an image. Lactic, acetic, and propionic acids required 5 to 10 hr., 2 to 3 days, and 3 to 4 days, respectively, to produce discernible images and even much more time for their full development. Wood sections left in acetic acid for 15 months were not seriously overdeveloped.

While KenKnight found that the acid treatment works with both root and stem tissue best results were obtained by using roots more than 1 cm. in diameter. Stem wood sections were more variegated, and the development of the dots in transverse sections required a longer time and the dots were fewer in number and smaller so that accurate reading required a hand lens or a dissecting microscope. In the summer, stem wood of the current season growth was less likely to give a positive phony reaction than older wood. In all cases the dots were confined to the xylem. Because it was easier to ascertain absence of blemishes in stems than in roots, there is some advantage in using stems for acid testing if good results can be obtained. Stems 5 to 10 mm. in diameter were satisfactory although stems of larger size could be used. Although Hutchins originally suggested a minimum of ten scattered dots for a positive reading, fewer dots appear to suffice for a significant determination.

Colorimetric tests.—In a series of studies on the chemistry of symptom production, Resühr (5) was able to produce effects simulating many of the symptoms reported for mottling type viruses. Using a 2 to 4 per cent solution of hydrolysable tannin injected in the shoot axis in doses of 0.05 to 0.1 ml. he reported the production of vein clearing, vein banding, mosaic mottling, and various other signs. He used bean, tomato, soybean, tulip, tobacco, rape, po-

tato, and other plants. Even as with the virus disease, several days were required before the simulated signs of disease appeared. The author concluded that many of the effects of viruses result from disturbances of the phenol and tannin metabolism of the host.

Lindner (6) devised a chemical test by which an alkaline extract of certain virus-infected peach and cherry leaves produced a brilliant red coloration. It seemed that this might be helpful in the diagnosis of virus-infected trees. Leaf discs, approximately 6 mm. in diameter, cut out with a paper punch or cork borer were placed in a ½-in. test tube which was standardized for use in a photoelectric colorimeter. Best results were obtained when these discs were cut from a leaf about midway between the tip and base and about midway between the midrib and edge of the leaf margin. To each test tube containing a leaf disc was added 5 ml. of reagent. This was then heated in a boiling water bath for 5 to 10 min., allowed to cool for 10 min., thoroughly shaken and read in a photoelectric colorimeter. The color reached its maximum in 10 min. and remained constant for at least 30 min. The reagent consisted of the following substances: 40 gm. sodium hydroxide, 0.3 gm. cupric sulfate and 3 gm. sodium citrate, dissolved in 1000 ml. of distilled water. The hydroxide was dissolved in one portion of water and the cupric sulfate and sodium citrate in another. The two solutions were mixed after the materials were completely dissolved.

Discs from normal leaves show a blue-green to yellow-green coloration. Virus-infected leaves give varying intensities of yellow to red. The test can be used for direct visual examination but a photoelectric colorimeter permits finer determinations and makes it possible to separate several different viruses on either cherry or peach. Using pure chemicals Lindner obtained indications that the color was probably due to polyhydroxy phenols and possibly to the tannin group.

A year later Kelly (7) stated that Lindner's test was merely a modification of the Benedict test for reducing substances. She assumed that the copper sulfate in the alkaline solution of Lindner's reagent was reduced to cuprous oxide, thus accounting for the red color produced. Using Lindner's reagent she obtained pronounced red coloration with glucose and virus tumor tissue, and less brilliant red coloration with agar on which the tumor tissue was grown and on virus-free pea leaves.

Later, Lindner *et al.* (8, 9, 10) provided further information on their color studies and concluded that virus infection induces the accumulation of phenolic compounds in the affected tissues. They maintain that the small amount of copper sulfate added to the reagent for the colorimetric test only serves to catalyze the oxidation of the polyphenols and is not essential to color formation. Their staining procedure consists of removing the chlorophyll of affected leaves, fixing the polyphenols, and developing blue colored polyphenol compounds with sodium hydroxide. Whole leaves or sections of the leaves are used. Decolorization and fixing are performed in one operation by the use of a reagent composed of 700 ml. of 95 per cent ethyl alcohol,

20 ml. of 37 per cent formaldehyde, and 230 ml. of distilled water. Samples can either be boiled under a condenser or heated in a water bath at 80°C. Chlorophyll from young leaves is removed in 5 to 10 min., but older leaves take as long as 2 hr. After decolorization, samples are transferred to normal sodium hydroxide and heated at 80° to 100°C. until maximum color develops. This time usually varies from 2 to 10 min. The development of a blue color indicates a positive reaction between polyphenols and sodium hydroxide. In the absence of polyphenols a yellow color develops. The blue coloration of leaves treated by this method is not permanent; the leaves become red through oxidation when exposed to air for 5 to 10 min.

It was noted that good staining reactions were obtained with those plant species having a polyphenol system similar to that present in deciduous fruit trees such as peach, cherry, apricot, plum, apple, pear, strawberry, rose, and high bush cranberry. Negative results were obtained with all virus-infected herbaceous plants tested except strawberry.

The authors pointed out that any type of phloem blockage such as girdling may produce phenol accumulation. The phenolic stain technique makes it possible to distinguish certain virus infections from other virus-like symptoms due to genetic variegations and injuries. The authors believe that the phenolic staining reaction is based upon a partial or complete blockage of the phloem or to the precipitation of the virus protein and polyphenol. If the latter is true, the staining reaction may be a test for the localization of inactivated virus particles.

Miller & Aldrich (11) carried out an extensive series of tests with the Lindner staining technique for detection of viruses in strawberries. More than 500 plants including 3 species of wild strawberries, 8 varieties of cultivated strawberries and over 25 unnamed hybrids were included. They used, with only minor modification, essentially the same procedure as described by Lindner *et al.* While definite positive stain reactions were always obtained in known cases of severe infection with yellows or crinkle, conflicting color reactions were obtained on leaves from plants mildly affected with yellows and with crinkle. In the acse of leaves from seedlings of Marshall strawberry, red pigment developed in the leaf cerations and light pink coloration in the lamina; this indicated that the plants were infected with virus. On the other hand grafts to "tester" plants showed the plants to be virus-free. The fact that sometimes positive results were obtained with virus-free plants introduces a complicating factor which detracts somewhat from the usefulness of the test especially in so far as strawberry disease investigations are concerned. The authors agreed that the Lindner test may have some value as a fast method for separating severely infected plants from those less severely diseased, and it may have some value in diagnosing the relative degrees of infection in a given lot of diseased plants. It did not appear to these investigators, however, to be a reliable method for separating healthy from infected plants.

Phloroglucinol tests.—Current season symptoms of leaf roll of potatoes is

characterized in many varieties by the production of a netted necrosis in the tubers. Sheffield (12) found that plants infected with leaf roll show a type of phloem obliteration which is distinct from any abnormality produced by other pathogens and from physiological causes. In potato leaf roll necrosis occurs only in the primary phloem of the bicollateral bundles. Quanjer *et al.* (13) and Esmarch (14) suggested that the substance produced in the necrotic cells was probably lignin. Brehmer & Rochlin (15) suggested that this material was suberin, and Esau (16) suggested that the material was a wound gum. In unstained condition the affected cells were seen to be of a yellowish-brown color. They stained readily with those dyes which are commonly used for lignified tissue such as safranin or malachite green, and they also reacted with phloroglucinol. These tests and reactions have indicated to many that the tissue is therefore lignified. It was pointed out that these stains are specific for lignin and a red color with phloroglucinol in HCl merely shows the presence of a pentose.

Sheffield used phloroglucinol to stain necrotic phloem cells found in potatoes affected with potato leaf roll. The stain was found to enhance the appearance of phloem cells showing necrosis. Wilson (17) used the phloroglucinol test as an aid in diagnosing leaf roll in Tasmania. This test helped distinguish questionable plants showing rolling of the leaves as a result of varietal character or fungus infection.

Klostermeyer (18) used this method successfully to diagnose current season infection in the Netted Gem variety. He devised a method for rapid sampling and examination. Samples were taken in the field by cutting off the top 6 in. of the stem from plants to be tested. Leaves were trimmed off and the stems held under refrigeration until they could be examined. Cross sections about ½ mm. in thickness were cut by hand through two nodes of the stem and stuck on an insect pin. These were immersed in a solution of 1 per cent phloroglucinol and alcohol for about 1 min., after which they were transferred to concentrated hydrochloric acid until the xylem turned red in color. After rinsing in water, the sections were examined at 40X magnification with a dissecting microscope. In healthy plants only the xylem and some fibers stained red, whereas in plants with leaf roll areas in the outer and inner phloem regions stained yellowish-red and were readily apparent.

Iodine, phenol, and methylene blue tests.—In 1931 Holmes (19) showed that the location of primary lesions of tobacco mosaic and the approximate path taken by the virus in leaving the inoculated area could be demonstrated by staining infected leaves with iodine. Leaves were immersed in 95 per cent alcohol until the chlorophyll was removed, stained in aqueous solution of iodine in potassium iodine for some 3 hr. or more, and finally rinsed in water to remove excess iodine. The test was based upon the differential rate of starch synthesis and hydrolysis in virus-infected tissue. Wartenberg & Klinkowski (20) used the iodine test to attempt separation of "degenerate" and healthy potato tubers. In their tests equal quantities of expressed juice from the two types of tubers were added to starch solution and mixed with

iodine in test tubes. After about an hour the entire contents turned blue in the case of healthy tubers, whereas in the case of the diseased tubers only the sediment at or near the bottom of the tube was discolored.

Hirata (21) used a modified iodine test to detect virus-infected potato tubers. A mixture of 0.5 ml. tuber juice and 0.5 ml. starch paste was added to 1 to 5 ml. of iodine solution (2 gm. in 100 ml. 30 per cent ethyl alcohol). The blue color faded in 50 min. if juice from virus-infected tubers was used, whereas mixtures containing juice from healthy tubers required more than 3 hr. to fade. The test was carried out with tubers of the Benimaru variety and was 70 per cent accurate if used immediately after harvest but much less dependable if used after tubers had sprouted.

Wartenberg & Lindau (22) attempted an analysis of the "dehydrase reactions" of healthy and "degenerate" potato tubers. The rapid clarification of the juice in contact with methylene blue solution indicated that the juice was from a "degenerate" tuber. They believed that such differences were due to the oxidation-reduction potentials of tissue emulsions and variations in their dehydrase reactions.

Lojkin & Beale in 1944 (23) presented the results of some quantitative microdeterminations obtained with the the aid of a Klett-Summerson photoelectric colorimeter using Folin phenol reagent for tyrosine, tryptophan, and cysteine derived from extracts of diseased plants. They were able to detect differences between one of the strains of tobacco mosaic virus and the other four strains.

Paper Chromatography

Using the method of paper chromatography described by Consden *et al.* (24), Cochran (25) described a variation of this method that may be used to test for tobacco mosaic virus. The success of the test depends upon the use of a solvent system that will move the virus to a given spot on the paper while not moving normal arginine-containing proteins to that same spot. He reported that, using water buffered at a pH range of 4.5 to 7.0 as the solvent system, it was possible to isolate spots on the paper containing highly active concentrations of virus free of chlorophyll or the normal arginine-containing proteins.

Ultraviolet Light

Holmes in 1928 (26) made a series of photographs of extracts obtained from various plants infected with viruses using ultraviolet as a light source. He found no formed structures other than those seen in corresponding fluids from healthy plants. In 1944 McLean & Kreutzer (27) examined a number of potato tubers with ultraviolet light and attempted to find a correlation between the degree of fluorescence and infection with virus diseases. By noting the degree and location of fluorescence they found a fairly good correlation with the occurrence of certain virus diseases and virus disease complexes. In 1947 Harris (28) conducted studies to test further the usefulness of ultra-

violet light in detecting the presence of virus diseases in potato tubers. Using nine varieties all grown in northern Maine, he found no apparent correlation between fluorescence in the tuber and the appearance of virus diseases in plants grown from these tubers. To test further the accuracy of the ultraviolet light in detecting virus diseases, he set up an additional test involving only the leaf roll virus in several varieties. He found that a considerable percentage of healthy plants was produced by tubers that fluoresced under ultraviolet light. Tubers from 22 varieties taken from plants showing distinct leaf roll symptoms exhibited several types of fluorescence. More than 50 per cent of the tubers from leaf-roll-infected plants showed a slight vascular fluorescence. Although the removal of all fluorescencing tubers would greatly decrease the percentage of virus infected tubers in a seed lot, there are no data to justify the conclusion that by means of ultraviolet light examination all virus diseased tubers could be eliminated.

An investigation by Eicke & Bode (29) on the usefulness of observing diseased plant material under ultraviolet light indicated that while the alterations differed markedly in appearance under ultraviolet as compared with daylight, the method seves no useful purpose in diagnosis because the same picture is presented by more than one virus. Thomas *et al.* (30) used an extract of terminal shoots and obtained a high correlation between fluorescence under ultraviolet and tests by inoculation in carnation mosaic diagnosis.

Electrophoresis

It appears that Vinson & Petre were probably the first to report that plant viruses migrate in the electrophoresis cell. They reported in 1929 (31) that tomato mosaic virus migrates to the negative electrode at pH 4.76. In 1930 Takahashi & Rawlins (32) found that unpurified tobacco mosaic virus migrates between pH 4 and pH 9.

In electrophoretic studies of plant viruses, Frampton & Takahashi (33) attempted to distinguish the electrokinetic properties of plant virus proteins. Specific scanning patterns were obtained with extracts of healthy tobacco plants and by tobacco plants infected with tobacco mosaic, potato X, potato Y, and two strains of cucumber mosaic. These patterns were found not to be identical. Three patterns present in extracts of healthy tobacco plants were not affected in concentration or nature by the development of tobacco mosaic in the plant. Abnormalities in the scanning pattern of tobacco plants infected with tobacco mosaic virus, however, was correlated in time with the appearance of symptoms in the plant. No attempt was made to work with viruses which are not readily transmissible by mechanical means.

Extensive use was made of the electrophoretic technique in the study of plant virus proteins by Wildman *et al.* (34, 35, 36) and Meneghini & Delwiche (37). Singer *et al.* (38) showed that this technique may serve for the precise characterization of different strains of tobacco mosaic virus.

Electron Microscopy

Kausche, Pfankuch & Ruska (39) first studied tobacco mosaic virus with an electron microscope. Their virus preparations were purified by chemical treatment which resulted in considerable inactivation and aggregation of the virus particles. Stanley & Anderson (40) reported in 1941 on the photography of purified preparations of five viruses: tobacco mosaic virus, cucumber mosaic virus 3, cucumber mosaic virus 4, tomato bushy stunt virus, and tobacco necrosis virus.

Hillier (41) has reviewed the application of electron microscopy to the study of microorganisms and viruses. The following works have been selected because of their direct bearing on the subject of this review.

Steere & Williams (42) described a simplified procedure for the preparation of viruses for examination by the electron microscope. In their studies they used tomato bushy-stunt virus prepared by coagulation of the normal protoplasm constituent by preheating, adsorption of normal components on to diatomaceous earth, and centrifugation. Their illustrations demonstrate that this method with bushy-stunt virus at least yields excellent material for electron microscopy.

Other investigators who have used the electron microscope for the characterization and identification of the virus particles themselves include Sharp & Wolf (43) who investigated tobacco leaf curl virus, Larson *et al.* (44) who investigated viruses affecting the genus *brassica*, Stahmann and associates (45) who studied Wisconsin pea-streak 3, and Jensen & Gold (46) who studied a ring spot disease of orchids. Black (47) presented electron micrographs of sections cut through infected tobacco leaves which indicate that tobacco mosaic virus is often present in fibrous masses associated with chloroplasts that are severely affected.

Oster & Stanley (48) in 1946 made electron microscopic studies of virus particles obtained directly from hair cells of tobacco plants infected with tobacco mosaic virus. Leaf hairs were clipped off, placed on a glass slide, and gently broken with a glass rod. The extruded contents were immediately transferred to electron micrograph screens with a drop of distilled water. Good particle figures were obtained.

Johnson (49) applied water pressure to plants in such a way that abundant guttation drops were produced. Studies were made on about 50 plant species infected with about 35 distinct viruses. Rodlike or threadlike particles were found associated with plants infected with several distinct viruses. Rods were clearly absent in guttation drops obtained from plants infected with a number of other viruses. The results suggested to Johnson that the rodlike particles may be the result of disease rather than the ultimate causal unit.

Electron micrographs of juice from *Nicotiana rustica L.* infected with potato yellow-dwarf virus by Black (50) revealed short rodlike objects which may be elementary particles of the virus. This is noteworthy because it

could be the first evidence of particulate nature in a plant virus transmitted by leaf hoppers.

Intracellular Inclusions

The presence of inclusions in cells of infected tissue was first noted by Iwanowski in 1903 (51). Bawden (52) and Cook (53) have presented complete reviews of the literature on intracellular inclusions. Those papers dealing with the use of inclusions for identification of virus infected tissue will be given special attention here.

Although inclusion bodies can sometimes be identified in hair cells without the use of stain, McWhorter (54, 55, 56) described a staining method using trypan blue which has proven useful in plant virus differentiation. The stains used are 0.5 per cent and 0.05 per cent trypan blue and 0.5 per cent phloxine made up in physiological salt solution (0.85 aqueous sodium chloride). Best results are obtained when a suitable wetting agent or detergent such as Vatsol (known as O.T. 100, made up in a 1 per cent solution dissolved in hot water) is used to help avoid air bubbles and to insure uniform penetration of stains.

When epidermal peelings from leaves are treated with trypan blue for 15 to 30 min., nuclei assume a light blue color and inclusion bodies, if staining occurs, become intensely blue. Chloroplasts remain green, and starch grains and oil globules are not stained.

When the combination stain is used, the epidermal peeling is placed in phloxine for 3 to 8 sec. and then washed briskly in physiological salt solution. The peeling is then transferred to the trypan blue for at least 2 to 4 min., rinsed, and mounted in physiological salt solution. If the stains balance properly the nuclei are deep blue and the inclusion bodies pink or purplish according to their ability to absorb the stain. The chief value of the combination stain technique is to make discernible those inclusion bodies that do not absorb trypan blue when used alone.

McWhorter believes that the method greatly facilitates the detection of "viroplasts," a term he proposed to designate cytoplasmic masses or "bodies" associated with or resulting from the presence of virus within a cell. He suggests that selective stains might become of specific use in the classification of plant viruses.

McWhorter used the trypan-blue technique for separation of tulip 1 virus from lily-latent virus and for distinguishing between virus infected tissue of *Pisum* virus 1 and tissues infected by *Pisum* virus 2 and *Phaseolus* virus 2. Using the same method McWhorter & Brierley (57) described the characteristics which distinguish lilies infected with lily rosette.

Using a prestain treatment of diethyl-ether-ethyl alcohol, Rich (58) found the trypan-blue method to be greatly facilitated.

Bald (59) described a method for selective staining of inclusion bodies produced in host cells by tobacco mosaic virus and other viruses. Pretreat-

ment with iodine-potassium iodide or concentrated acetic acid improved staining of the virus with Giemsa or Giemsa-Orange G. The tests were carried out mainly with strips of epidermal leaf tissue. In a later paper (60) he described the results obtained with a number of experimental fixative solutions including absolute alcohol, water, Lugol's iodine, formalin, glacial acetic acid, cerium nitrate, and chromic acid. Tissue samples were tested from plants infected with viruses of tobacco mosaic, potato-X, tomato spotted wilt, squash mosaic, potato leaf roll, and Pierce's disease in alfalfa. Some fixative solutions produced uniformly stained inclusion bodies and preserved the structure of plastids and other cell constituents that might be important in virus multiplication.

Summary

Although considerable effort has gone into attempts to develop reliable chemical means of diagnosing or identifying virus-infected plant tissues, reasonable success has been achieved in relatively few instances. This bears testimony to the complexity of the physiological effects of virus infection. More successful chemical tests, when achieved, will likely be specific in nature.

Electron microscopy has not only substantiated information regarding certain characteristics of viruses derived from other techniques but has added further important knowledge. Through the application of information derived from electrophoresis, serology, isotopic "tracer" techniques, as well as other chemical and physical studies, it seems possible that satisfactory tests for the identification and diagnosis of virus-infected tissues may be developed.

LITERATURE CITED

1. Hutchins, L. M., *Georgia State Entomol. Bull., No. 78*, 3–55 (1933)
2. Hutchins, L. M., Cochran, L. C., and Turner, W. F., *U. S. Dept. Agric., Agric. Handbook*, **10**, 17–25 (1951)
3. KenKnight, G., *Phytopathology*, **41**, 829–32 (1951)
4. KenKnight, G., *Phytopathology*, **41**, 20–21 (1951)
5. Resühr, B., *Z. Pflanzenkrankh. Palanzenschutz*, **52**, 63–68 (1942)
6. Lindner, R. C., *Science*, **107**, 17–19 (1948)
7. Kelly, S., *Science*, **109**, 573–74 (1949)
8. Lindner, R. C., Weeks, T. E., and Kirkpatrick, H. C., *Phytopathology*, **39**, 1059–60 (1949)
9. Lindner, R. C., Kirkpatrick, H. C., and Weeks, T. E., *Science*, **112**, 119–20 (1950)
10. Lindner, R. C., Weeks, T. E., and Kirkpatrick, H. C., *Phytopathology*, **41**, 897–902 (1951)
11. Miller, P. W., and Aldrich, F. D., *Plant Disease Reptr.*, **35**, 131–33 (1951)
12. Sheffield, F. M. L., *Ann. Applied Biol.*, **30**, 131–34 (1943)
13. Quanjer, H. M., Van der Leh, H. A. A., and Oortwijn Botjes, J. G., *Mededeel. Landbouwhoogeschool Wageningen*, **10**, 91–138 (1916)
14. Esmarch, F., *Monographien Pflanzenschutz*, **8**, 1–91 (1932)
15. Brehmer, W. von, and Rochlin, E., *Phytopath. Z.*, **3**, 471–98 (1931)
16. Esau, K., *Botan. Rev.*, **10**, 548–79 (1938)
17. Wilson, J. H., *J. Australian Inst. Agr. Sci.*, **14**, 76–78 (1948)
18. Klostermeyer, E. C., *Plant Disease Reptr.*, **34**, 36–38 (1950)
19. Holmes, F. O., *Contribs. Boyce Thompson Inst.*, **3**, 163–72 (1931)
20. Wartenberg, H., and Klinkowski, M., *Phytopath. Z.*, **10**, 107–9 (1937)
21. Hirata, S., *Ann. Phytopath. Soc. Japan*, **14**, 25–28 (1950)
22. Wartenberg, H., and Lindau, G., *Phytopath. Z.*, **9**, 297–324 (1936)
23. Lojkin, M. E., and Beale, H. P., *Contribs. Boyce Thompson Inst.*, **13**, 337–54 (1944)
24. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224–32 (1944)
25. Cochran, G. W., *Phytopathology*, **37**, 850–51 (1947)
26. Holmes, F. O., *Botan. Gaz.*, **86**, 59–65 (1928)
27. McLean, J. G., and Kreutzer, W. A., *Am. Potato J.*, **21**, 131–36 (1944)
28. Harris, M. R., *Am. Potato J.*, **24**, 179–83 (1947)
29. Eicke, R., and Bode, O., *Arch. ges. Virusforsch.*, **3**, 327–34 (1947)
30. Thomas, W. D., Jr., Baker, R. R., and Zoril, J. G., *Science*, **113**, 576–77 (1951)
31. Vinson, C. G., and Petre, A. W., *Botan. Gaz.*, **87**, 14–37 (1929)
32. Takahashi, W. N., and Rawlins, T. E., *Hilgardia*, **4**, 441–63 (1930)
33. Frampton, V. L., and Takahashi, W. N., *Phytopathology*, **36**, 129–41 (1946)
34. Wildman, S. G., and Bonner, J., *Phytopathology*, **40**, 31–32 (1950)
35. Wildman, S. G., Cheo, C. C., and Bonner, J., *Biol. Chem.*, **180**, 985 (1949)
36. Wildman, S. G., and Bonner, J., *Sci. Monthly*, **70**, 347–51 (1950)
37. Meneghini, M., and Delwiche, C. C., *J. Biol. Chem.*, **189**, 177–86 (1951)
38. Singer, S. J., Bald, J. G., Wildman, S. G., and Owen, R. D., *Science*, **114**, 463–65 (1951)
39. Kausche, G. A., Pfankuch, E., and Ruska, H., *Naturwissenschaften*, **27**, 292 (1939)
40. Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, **139**, 325–38 (1941)
41. Hillier, J., *Ann. Rev. Microbiol.*, **4**, 1–20 (1950)
42. Steere, R. L., and Williams, R. C., *Phytopathology*, **38**, 948–54 (1948)

43. Sharp, D. G., and Wolf, F. A., *Phytopathology*, **39**, 225–30 (1949)
44. Larson, R. H., Matthews, R. E. F., and Walker, J. C., *Phytopathology*, **40**, 955–62 (1950)
45. Stahmann, M. A., Hagedorn, D. J., and Burger, W. C., *Phytopathology*, **40**, 999–1006 (1950)
46. Jensen, D. D., and Gold, A. H., *Phytopathology*, **41**, 648–53 (1951)
47. Black, L. M., *Proc. Soc. Exptl. Biol. Med.*, **73**, 119–22 (1950)
48. Oster, G., and Stanley, W. M., *Brit. J. Exptl. Path.*, **27**, 261–65 (1946)
49. Johnson, J., *Phytopathology*, **41**, 78–93 (1951)
50. Black, L. M., *Biochim. Biophys. Acta*, **2**, 121–23 (1948)
51. Iwanowski, D., *Z. Pflanzenkrankh.*, **13**, 1–14 (1903)
52. Bawden, F. C., *Plant Viruses and Virus Diseases*, 2nd Ed. (Chronica Botanica Company, Waltham, Mass., 290 pp., 1943)
53. Cook, M. T., *Viruses and Virus Diseases of Plants* (Burgess Publishing Company, Minn., 244 pp., 1947)
54. McWhorter, F. P., *Phytopathology*, **30**, 788 (1940)
55. McWhorter, F. P., *Stain Technol.*, **16**, 143–49 (1941)
56. McWhorter, F. P., *Phytopathology*, **31**, 760–61 (1941)
57. McWhorter, F. P., and Brierley, P., *Phytopathology*, **41**, 66–71 (1951)
58. Rich, S., *Stain Technol.*, **23**, 19–20 (1948)
59. Bald, J. G., *Phytopathology*, **39**, 395–402 (1949)
60. Bald, J. G., *Am. J. Botany*, **36**, 335–42 (1949)

NEMATODES PARASITIC ON PLANTS[1]

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The subject of nematode diseases of plants has undergone extremely rapid, even revolutionary, development within the last decade. Although the existence of a phytoparasitic nematode, the wheat eelworm, was recorded by Needham (178), and both Bastian (12) and de Man (165) included phytoparasites in their extensive monographs, actual investigations of these forms and diseases they cause began only in the late 1880's, such work being done by horticulturists and phytopathologists such as Kühn, Bos, and Atkinson. In 1907 Cobb set up, for the first time, an organization in the U. S. Bureau of Plant Industry for the study of these forms.

Textbooks on phytoparasitic nematodes were published by Goodey (112) and Filipjev & Stekhoven (90). In this review we shall attempt to point out the major developments of the field since the latter text or, more properly speaking, since December, 1939. As of that date a number of plant diseases were known or thought to be caused by nematodes. While the techniques of artificial inoculation and transfer had at times been resorted to in the study of such diseases, they could hardly have been called standard practice. We might well say that the mode of experimental procedure had not been properly developed. Likewise chemical treatment of soils had been studied experimentally but had not been brought to a sound basis. The only therapeutic measure developed up to that time was the application of hot water in the treatment of such diseases as stem and bulb nematode of narcissus. Considerable work had been done, from a field transfer standpoint, toward the recognition of strains or races of several plant pathogenic nematodes. Extensive host lists of a few of the major pathogens had been prepared and these commonly contained evidence of heterogeneity in the species concept. In January, 1940, we might well have said the phytonematologist was in a little better position than he was in 1930. At that time he could learnedly tell the farmer or horticulturist the names of some organisms found in a diseased plant and indicate that the plant would probably be better off without said organisms. He could seldom swear, or affirm, that one of the organisms was truly pathogenic nor could he tell his customer how to get rid of the organisms. In substance, he could only suggest that the grower would do well to plant clean plants on clean land, neither of which he himself could pro-

[1] The survey of the literature pertaining to this review was concluded in February, 1952.

vide. This is undoubtedly an exaggeration since crop rotation, horticultural breeding, and natural selection had been in progress for several years. Even so, we feel the reader will understand that the position of phytonematology was not financially or scientifically sound.

The past 20 years have seen great strides in the development of many phases of the subject. Goodey (112) lists 363 references on phytoparasitic nematodes. Filipjev & Stekhoven (90) list approximately 2500 articles on the same subject. Since 1941, the bibliography on phytoparasitic nematodes has increased to approximately 3400 references. These figures may be a bit misleading since Filipjev & Stekhoven list only 165 articles on phytonematology from 1933–1940 inclusive and the references which these authors have added include not only older literature on plant pathogens, plant associates, but also many more or less extraneous articles. The past 10 years have seen quite an upsurge in publications despite intervening war years. Even though our bibliographies of those years are still quite incomplete, no less than 900 articles are listed between 1940 and 1951. Analysis shows that many of these articles are in the nature of local press, farmers bulletins, and other publications of a popular or semipopular nature. Even so, there has been a marked increase in constructive papers. In fact, we find ourselves faced with the necessity of omitting many articles containing useful information from the present review. In general, we will limit our references to those which seem to have led the way in early stages of investigations or to those which have brought out points conclusively for the first time.

TAXONOMY, HOST RANGE, AND BIONOMICS

The taxonomy of phytoparasitic nematodes has passed through several phases including considerable lumping and splitting. The natural desire of taxonomists and pathologists to define the various organisms in such a way as to permit prediction of behavior has met with considerable difficulty. Micoletzky (172) was obviously quite impatient with such aims and lumped together many forms which were rather well defined. Goodey (112) presented a more useful monograph of phytoparasitic nematodes but he was extremely conservative both in the generic and specific sense. Filipjev (89) and Filipjev & Stekhoven (90), purely on the basis of literature, took major steps in the separation and recognition of genera and species. Intervening years have proved that most of their separations were sound though the evidence at the time was inadequate. Thorne (245) and Goodey (116) have revised the classification of the genera occurring as nematode parasites of plants.

In each of our major phytoparasitic nematode genera, earlier authors have described organisms found in or associated with a given plant with certain symptoms as species. Later they themselves or others found similar organisms associated with other plants under natural conditions and considered them the same species. Few controlled inoculations under sterile or semisterile conditions were made. Hence a mass of confusing data was placed on the record. In recent years we have realized the inadequacy of prior knowl-

edge, and with this recognition has come an extended effort to obtain more reliable information.

The Genus Heterodera Schmidt

The genus *Heterodera* includes several of the best known plant pathogens occurring in the roots of agricultural crops. Formerly it included the root-knot nematodes but these have recently been recognized as a separate genus *Meloidogyne*. As presently constituted, the genus *Heterodera* contains more or less distinctly ectoparasitic nongall-producing root parasites in which the female is transformed into a tough resistant cyst containing the eggs of the new generation. In this condition the organisms may lie dormant for a period of years but are reactivated in the presence of root leachings from certain plants. Apparently the stimulatory substance necessary is a more or less specific compound according to the particular nematode. For many years all of the organisms presently classified in the genus *Heterodera* were lumped together under the name *H. schachtii* Schmidt, 1871. Discrepancies in morphology and host range of parasites as found in agricultural crops led early investigators to seek explanations. Thus Liebscher (148, 149, 150) noted some structural differences between the pea nematode and the sugar beet nematode. He also conducted field plot tests in which peas and sugar beets were each planted continuously on adjacent plots for a number of years. Thereafter, a wide series of potential hosts were planted on each plot. The results were striking in that there was a wide separation of the host ranges in the two plots. On this basis Liebscher proposed a new specific name for the pea nematode, namely, *H. göttingiana*. Steiner (211), in reviewing this case, concluded that here was a distinct example of natural selection or the inheritance of acquired characters.

Mortenson & Kolpin (176) on the basis of host range studies named the oat nematode *Heterodera schachtii* var. *avenae*. Zimmermann (269) and Wollenweber (265) investigated the *Heterodera* of potatoes, concluding on the basis of host range and morphology that this form was also distinct from the sugar beet nematode. The former author regarded it as a variety for which he proposed the name *H. schachtii* forma *solani* while the latter author considered it a separate species, *H. rostochiensis*. Thorne (239) described a nematode from wheat, *H. punctata*. Schmidt (200) and Goffart (107) made biometrical studies on the *Heteroderas* of oats, beets, brassicas, and potatoes in an attempt to set up better criteria for the recognition of these forms. Filipjev (88) considered *H. schachtii*, *H. avenae*, *H. punctata*, *H. göttingiana*, and *H. rostochiensis* as separate species. However, these species were not recognized in Britain or the United States until Franklin (92) made more extensive biometrical and morphologic studies. Such investigations have been continued by Jones (135), Oostenbrink (183), and Fenwick & Franklin (86, 87). Regrettably these investigators have not combined critical or minute morphology with their other studies. From these investigations we have learned that the older host lists of the sugar beet

nematode were invalid. It appears at present as though there are several major types of *Heterodera* as based on cyst wall, cyst shape, larval length, tip of larval tail, position of dorsal gland orifice, and other characters. These types appear to breed true and to have specific host ranges. Unfortunately, the host ranges overlap in some instances and very similar forms with both a wide and a narrow host range may coexist in the same soil. Segregation of such forms as morphologic entities is a great step in the planning of rotation schedules and in the preparation of inoculum for testing of plants when breeding for resistance. Raski (192) presents very interesting information on the host range of *H. schachtii*. In some plants, i.e., sweet clover, castor bean, and *Crotalaria spectabilis*, larvae invaded the roots but were unable to undergo development, while in several other plants a few specimens reached maturity and produced eggs and larvae. The progeny were tested on both the original host, sugar beet, and on another generation of the transfer host. In most instances no development occurred on the second host, e.g., lupine, cowpea, golden wax bush bean, nightshade, and *Sesbania macrocarpa*. In some instances with transfers from *Sesbania*, no development was obtained even on sugar beets. These investigations certainly should cause reassessment of transfer testing techniques and indicate much more caution is necessary in the assignment of specific rank on the basis of past host range studies.

At the present time, the following species of *Heterodera* have been recognized though several are inadequately described or characterized:

1. *H. schachtii Schmidt*, attacks Chenopodiaceae and Cruciferae.
2. *H. göttingiana* Liebscher, attacks peas and some other Leguminoseae (evidence conflicting).
3. *H. trifolii* Göffart & Oostenbrink, attacks clover and Leguminoseae.
4. *H. avenae* Mortenson & Kolpin (176), attacks oats and other Graminaceae.
5. *H. cacti* Filipjev & Stekhoven (90), attacks *Phyllocactus* and *Cereus*.
6. *H. weissi* Steiner, attacks *Polygonum* species.
7. *H. carotae* Jones (135), attacks carrots.
8. *H. cruciferae* Franklin, attacks Cruciferae.
9. *H. punctata* Thorne (239), attacks *Agrostis* spp. and wheat.
10. *H. rostochiensis* Wollenweber (265), attacks potatoes, tomatoes, and certain other Solanaceae.
11. *H. galeopsidis* Goffart, attacks *Galeopsis tetrahit*.
12. *H. humuli* Filipjev (89), attacks hops.

The basic life history of these organisms was worked out by Strubell (220). More recently Raski (191) has made a thorough study of the life history of *H. schachtii* discovering a first molt within the egg shell, the second stage larva being infective. Hagemeyer (122) has made the same observation relative to *H. rostochiensis*.

Temperature relations as a factor in the interaction of parasite and host were first investigated by Miles (173), who concluded that a minimum

temperature of 15–16°C. is necessary for the activation of dormant larvae in cysts of *H. rostochiensis*. Chitwood & Buhrer (37) studied the relation of temperature to invasion of potato roots. They found no evidence of mass invasion during periods of a weekly mean temperature of 10°C. but distinct evidence of such invasion during periods of a weekly mean of 15°C. The rate of root growth in potato varieties at various temperatures is apparently a major factor in determining the damage sustained by these varieties as a result of the parasites. Feldmesser & Fassuliotis (83) found that some eggs hatched at 4°C., that winter cysts were more resistant to adverse conditions than summer cysts, and that overwintering was not essential to the life history of *H. rostochiensis*. Chitwood (35) reported that potato plants grown in constant temperature tanks showed no evidence of invasion at 12 and 13°C. but developed massive infections at 19°C. In this connection, Fenwick (85), on the basis of laboratory tests, described a series of experiments on the effect of some physical conditions on larval emergence from the cysts. The factors investigated were presoaking of the cysts, temperature, volume of diffusate per cysts, pH, dilution of diffusate, and sunlight. He also devised a shortened method for the conduct of hatching tests by making use of the fact that when larval emergence from a batch of cysts is plotted against log time, a symmetrical sigmoid curve is obtained. A geometric method of estimating the point of inflection of such a sigmoid curve is given in his paper and it is claimed that the number of larvae emerging at this point represents one half ot the total "hatchable" larvae under the conditions of the test.

The relation of numbers of cysts in soil to disease symptoms and to numbers of cysts present in the soil following a crop has been one of the most difficult problems for many years. Presence of fungi in heavily infested roots and difficulty in obtaining correlation of plant growth with nematode infection caused much confusion in earlier years. Carroll & McMahon (22, 23, 24) artificially infected pots with known numbers of viable cysts and by this means showed that disease symptoms could be produced and that the greater the quantity of inoculum the smaller the relative population increase. Johnson & Thompson (132) correlated viable cyst counts in the field with disease symptoms, finding crop damage usually occurred with viable cyst counts of four or more per 10 gm. of soil. More extensive studies were made by Chitwood, Feldmesser (35, 38) and by Oostenbrink (183). These workers investigated the relationships of population numbers to rate of increase, distribution of the disease organism, and crop damage. In general the population at a given locus was found to increase logarithmically, the rate of increase leveling off as crop damage begins and declining severely when the crop damage is pronounced. Extremely high populations may cause destruction of the root system with a consequence that the net number of nematodes per unit of soil is reduced. The total population tends to come to a dynamic equilibrium which may be termed the saturation level. This level varies from year to year according to the growing season, varieties planted,

and other environmental conditions. Local spread in the field is, of course, a function of the numerical population. Means of spread include farm implements, drainage water, wind, potatoes, soil adhering to other root crops, and the containers used for marketing crops. While these points have been worked out for a specific nematode, they are more or less applicable to most root parasitic nematodes.

The Genus Meloidogyne

This group commonly called the "root-knot nematodes" was formerly considered a single species placed in the genus *Heterodera*. Over a period of years they have been known as *Heterodera radicicola*, *Caconema radicicola*, and *Heterodera marioni*. An annotated host list was prepared by Tyler (258) showing many conflicting reports as evidenced by miscellaneous field observations and by discrepancies in the results of plant breeders seeking to develop resistant varieties. These discrepancies led Christie & Albin (51), Christie (47), and Christie & Havis (55) to conduct controlled studies on the host range of various root-knot populations. These workers were able to segregate several distinct "races" or "populations" which showed overlapping but distinctive host ranges. Chitwood (34) described several of these forms morphologically and further studies on the host ranges have been conducted by Chitwood, Specht & Havis (40). Unlike the genus *Heterodera*, species of the genus *Meloidogyne* have wide host ranges so that a full listing may require many years. Despite these wide host ranges, host specificity and various degrees of resistance may exist in a given species of plant. In this connection Sasser (197) showed that the several different species of the genus *Meloidogyne* could be identified on the basis of host reaction alone. The presently named species and subspecies of the genus *Meloidogyne* and their host ranges are listed as follows:

1. *M. marioni* Cornu. Type host: *Onobrychis sativa*, Chateauneuf sur Loire, France. Inadequately known.
2. *M. exigua* Goeldi. Type host: *Coffea* sp., Rio de Janeiro, Brazil.
3. *M. javanica* Treub. Type host: *Saccharum officianarum*, Java. Reproduces on Lovell, Yunnan, and Shalil peach roots; does not reproduce on peanuts.
4. *M. incognita* Kofoid and White. Type host: Man, substitute host carrot, Texas. Reproduces on Lovell peach roots, yellow globe onions, and cotton but does not reproduce on peanuts, *Lycopersicon pervianum*, or Yunnan and Shalil peach roots.
5. *M. incognita acrita* Chitwood. Type host: Cotton, Tifton, Georgia. Reproduces on Lovell peach, also on *Lycopersicum pervianum* but not on Yunnan or Shalil peach root stocks.
6. *M. arenaria* Neal. Type host: Peanut, Archer, Florida, or Lake City, Florida, Does not reproduce on Lovell, Yunnan, or Shalil peach root stocks.
7. *M. arenaria thamesi* Chitwood. Type host: *Boehmeria utilis*, Canal Point, Florida. Does not reproduce on peanut.
8. *M. hapla* Chitwood. Type host: *Solanum tuberosum*, Long Island, New York. Reproduces on peanuts with smaller galls than *M. arenaria*. Does not re-

produce on Lovell, Yunnan, or Shalil peach root stocks, and reproduces very poorly on yellow globe onions. Only species known from strawberries.

9. *M. vialae* Lavergne, n. Comb. Syn. *Anguillula vialae* Lavergne. Type host: Grape vine roots, Chile. Inadequately described.

Apparently unknown or undetermined host-parasite physiology is related to genetics and biochemistry more closely than to plant taxonomy. Fundamental studies to determine the nature of these relationships will provide problems for many years to come. Through the segregation of species and subspecies we have only opened the door to basic research. What the future holds in store for the investigators of host-parasite biochemistry and parasite variants such as mutations, cannot be foretold.

Tyler (256) showed that at least some root-knot nematodes are capable of reproduction from single larvae indicating that development is either parthenogenetic or hermaphroditic. According to her investigation, males are less commonly observed in light than in heavy infestations but the pedigree charts indicate rather erratic appearances of males. It seems possible that parthenogenetic and bisexual development may occur simultaneously in some root-knot nematode species. Variances in perineal patterns of females (34) and in the occurrence of intersexes certainly provide an interesting problem for solution.

Since much of the basic work on root-knot nematodes long preceded our present specific concepts, we have no means of attributing this knowledge to particular species. However, the species are in general so similar that differences, though important, are probably very small in most cases. The eggs are deposited in a fibrous mucoid mass and one molt occurs within the egg shell according to Christie & Cobb (53). Other stages in the life history are well presented by these authors and also by Nagakura (177). Since temperature has a direct influence on the rate of nematode metabolism, it is obvious that it must have an important bearing on many problems, such as rate of travel through soil, rate of killing by chemicals, and rate of starvation in fallow fields as well as the amount of infection and damage done to crops, which have been investigated by Godfrey (98) and Jones (136). Tyler (257) made an extensive study on the development of the root-knot nematode as affected by temperature. She indicated that the minimum time required for the life cycle of the root-knot nematode from larva to larva in experiments in tomato roots was 25 days at 27°C., increasing to 87 days at 16.5°C. Development from gall formation to egg-laying required 15 days at 27°C. and 79 days at 14.3°C. Temperature is not, however, the only factor influencing the rate of development of this nematode. Godfrey & Oliveira (104) grew cowpea and pineapple plants side by side in the greenhouse, yet under identical conditions, development to egg-laying took 35 days in pineapple and only 19 days in cowpea. They indicated that such difference appeared to be inherent in the two host plants. Oteifa (186) also indicated that the duration of the life cycle of the root-knot nematode depends on the nutritional status of the host plant. A great deal of significance has been attributed to the particular

giant cell formation causing the gall symptoms. These have been investigated histologically by Tischler (250), Kostoff & Kendall (138), and Christie (41). The latter author found these structures to result from the hypertrophy and coalescence of undifferentiated cells otherwise destined to be of vascular nature. This effect is apparently due to the injection and diffusion of nemic salivary enzymes. The nematode remains sedentary, feeding on the adjacent giant cells which have been fancifully called "nectaries."

Observations on varietal differences in resistance to root-knot in economic plants have been recorded by many investigators. For the purpose of this review we will attempt to illustrate some examples of plants reported as exhibiting resistance to root-knot. Tufts & Day (254, 255) in California reported that seedlings of the Shalil and Bokhara peaches were practically "immune" to root-knot while the Hony and Saucer types were somewhat less susceptible than the average peach. Later tests indicated that the Bokhara seedlings were not so resistant as previously supposed [Currie (70)], which, together with the fact that they are relatively "shy bearers," has led this variety to be disregarded as a possible rootstock for general use. Seedlings of certain introductions from Yunnan Province, China, also are reported to show resistance. Recent studies by Chitwood, Specht & Havis (40) proved that the different varieties of peach reacted differently to the several root-knot nematode species tested but a given variety also exhibited varied reactions according to quantity of inoculum and duration of infection or plant growth. Poole & Schmidt (189) working with sweet potatoes in North Carolina found the Jersey and Puerto Rico varieties to be highly resistant, while Yam, Yellow Yam, Southern Queen, Red Bermuda, Nancy Hall, and other varieties were susceptible. Malloch (164) worked on the problem of breeding nematode resistant tomato plants. He found 98 tomato varieties and eight hybrids to be susceptible, but made no attempt to distinguish degree of susceptibility. Christie & Albin (51) and Chitwood, Specht & Havis (40) have pointed out cases of host specialization and host preference among different populations. Cases have been known where certain plants appeared resistant to one nematode species and susceptible to another.

In discussing the problem of the nature of root-knot resistance, Barrons (11) indicated that larvae enter the roots of certain resistant plants at as great a rate as they enter susceptible plants. He stated that resistance therefore appears to be the result of something within the plant that either kills the larvae or counteracts their gall inducing effect. In this regard we feel students of host resistance should be aware of some of the basic concepts of plant immunology.

The problem of resistance and susceptibility has been a source of much confusion both as to nomenclature and facts. The interrelationships of plant nematodes and their hosts involve practically every imaginable degree of parasitism and specificity. Attempts by practical agriculturists to solve problems and to publish observations on varietal testing have resulted in chaos as far as the meanings of their words are concerned. More specific in-

formation as to the true nature of the "resistance" involved will be necessary before much of the data can be evaluated.

Because of the extreme difficulty in defining these interrelationships, Christie (50) preferred to classify plants as "suitable" or "unsuitable" hosts. Following this nomenclature, an unsuitable host would be one in which the larvae either would not invade, or having invaded, would be unable to complete their life cycles. Causes of this unsuitability might be highly varied: (*a*) absence of an attracting root diffusate; (*b*) thick cell walls or cell walls chemically resistant to nemic enzymes; (*c*) presence of chemical inhibitors or poisons in the plant cells: (*d*) absence of necessary response on the part of the plant, specifically, lack of giant cell formation; (*e*) absence of necessary nutrients required for nemic development; (*f*) death of plant cells as a result of over-susceptibility to nemic enzymes.

The antithesis, namely a "suitable" host, would be one in which the nematode would reproduce well without necessarily causing much damage. From the standpoint of horticulturist or pathologist, such a host would best be defined as tolerant. Degrees of tolerance are numerous and depend on extraneous factors such as moisture, temperature, and mineral content of the soil. Under some circumstances a tolerant host is capable of maintaining itself without marked reduction in growth rate or yield and may even be stimulated, and increased growth may result from the nematode infection. An immune plant may be defined as one to which the nematode is either: (*a*) not attracted to enter; (*b*) having entered, is wholly incapable of further development. The cause of this immunity may be one of many, but in the case of invasion, the result is either necrotic areas (oversusceptibility) in the area around the nematode as mentioned by Christie (50), or special pathologic responses, for example, liquification in the area of the parasite. Immune plants may be overwhelmed by superinfection with a given nematode. Such mass infections can and do undoubtedly cause considerable hardship as indicated by practical root destruction in some cases, but if the plant is capable of surviving the initial damage through favorable growing conditions, it may completely outgrow the initial setback.

Resistant plants are plants to which the nematodes are attracted and are able to enter but which are resistant because the parasites do not reproduce well; hence they are "unsuitable" hosts in the sense used by Christie. Various degrees of resistance make the terminology difficult. A very highly resistant plant may be defined as one in which only an occasional parasite produces young. This degree of relationship is much more commonly encountered than immunity. The parasite population is reduced under such circumstances. Causes of resistance are often obscure. They may fall under headings grouped as follows: (*a*) failure of the plant to provide proper nourishment for the parasite, as often indicated by lack of giant cell formation, or (*b*) formation of tissues comparable to scar tissue or cyst formation in animals. The latter circumstance in animals would be classified as an immunological response. Specifically, an abnormal histological reaction as a result

of nemic enzymes or other foreign body may cause parasites to be literally walled off from nourishment. An example is *M. hapla* in the roots of yellow globe onions mentioned by Chitwood (35).

Population Dynamics and Plant Growth

Population dynamics is closely integrated with resistance and tolerance studies. The numbers of nematodes as measured by either number of larvae or numbers of egg masses used as inoculum of a given plant variety of stated size directly determines the effect of the nematode on the rate of growth and even the chemical constitution of the plant as pointed out by Chitwood, Specht & Havis (40) and Oteifa (186). The interaction of these various factors also determines the numbers of nematodes produced to act as inoculum for the new quantity of plant after any given time lapse. Tarjan (225) has found varied rates of reproduction according to numbers of nematodes in inoculum of a given species with a given variety of snapdragon. Oteifa (185) showed that differences in the number of females of the nematode *M. incognita* produced on the roots of lima bean plants and difference in the rate of their oviposition can be induced by variations in potassium nutrition of the host plant. However, such differences in rate of reproduction varied with the numbers of nematodes in the inoculum. Reduction of root-knot nematode population as a result of overinfestation of susceptible, suitable, or even tolerant hosts can and does occur. The mechanism of such phenomena can best be understood by citing a case in which a lima bean varietal resistance test was a failure. Many seedlings both tolerant and susceptible failed to emerge. Those which did emerge gave a spotty poor stand. Many plants died before reaching maturity and the plants sent in for evaluation were so heavily infected that when one split the tap root, adult female nematodes (practically no eggs) fell out of the root tissue, like pearls from a broken string. Such dramatic demonstrations are rare but help to explain more obscure cases.

The proper quantity of the proper nematode with the correct variety of host sometimes stimulates the rate of not only root but also top growth as indicated by Chitwood (36), Chitwood, Specht & Havis (40). Such interactions are usually temporary but theoretically might be continuous in the case of an immune or very highly resistant perennial plant grown in conjunction with a moderately resistant plant which would serve to maintain a proper balanced inoculum for the perennial host.

The Genus Pratylenchus Filipjev

Members of the root parasitic genus *Pratylenchus*, or meadow nematodes, are migratory in the root cortex and suspected of being pathogenic. Actual proof of their pathogenicity is rather recent. Many species have been described by pathologists in various parts of the world and most of these were synonymized with *Pratylenchus pratensis*. Morphologic and host range studies are gradually proving the validity of these species. Early investigations

on the host range and life cycle of *Pratylenchus* species were made by Cobb (61), Rensch (193), Steiner (212), and Goffart (105). Unfortunately much of the information attributed to *P. pratensis* in the literature should probably be referred to *Pratylenchus* species. At present 14 species of *Pratylenchus* have been named.

Hastings & Bosher (125) showed by artificial inoculation that meadow nematodes are capable of reducing the growth rate of plants by 50 to 75 per cent. The lesions they cause are commonly found to be infected with fungi which tend to confuse the picture. Hastings (124) described the life history and reproduction in oats. He found that the life cycle required 54 to 65 days, the egg stage requiring a minimum of 29 to 34 days, the period required for four larval stages and pre-egg laying stage of the adult female being 25 to 31 days. He also observed that these organisms are unable to withstand desiccation. Gadd (96) and Gadd & Loss (97) have studied the life history and pathology of meadow nematodes to tea and *Tephrosia vogelii*. It is quite obvious that these investigators were not dealing with the same species as that of Hastings. Gadd & Loss (97) found the minimum incubation period of free eggs to be 12 days, while in roots a minimum incubation period of 16 days was obtained; the mean larval period was determined to be 15 days but egg laying of the new generation was not found to occur before the fifth week after deposition of an egg. The females apparently required fertilization and produced an average of 1.6 eggs per day for a period of not over five weeks. The same authors (97) studied the behavior of the adult female within seedling roots of *Crotalaria anagyroides*, *Desmodium gyroides*, turnip, and tea and compared it with its behavior in *Tephrosia vogelii*. They found that in tea roots as in *Tephrosia*, the worms settled down to a sedentary life; an average of 19 or more eggs were laid in three weeks and those hatched normally. In *Desmodium* roots, egg laying proceeded normally during the first week, but later diminished. An average of nine eggs only was laid in three weeks. Many worms left the roots after laying eggs. In *Crotalaria* and turnip roots few eggs were laid and many worms left without laying eggs. Few eggs hatched within *Crotalaria* roots or the larvae left soon after emergence from the eggs. They concluded that resistance of roots to attack by this nematode is not a resistance to entry. It is demonstrated by the departure of worms from invaded roots and an inability of the worms to reproduce at a normal rate. Cobb (61) recognized the importance of minute morphologic differences in this genus, but until Steiner (213 to 216) re-emphasized these differences they were overlooked despite the many perplexing problems that arose as a result of mixed infestations. These nematodes have been suspected of being a part of several "root rot complexes." Smith (205) and Taylor & Smith (233) found no correlation between meadow nematode injury and cotton wilt. Anderson (4), Jenkins (130), Valleau & Johnson (260), and Graham (120) established the fact that brown root rot of tobacco is caused by these organisms. The latter found two species in mixed infestations in tobacco roots, *P. leiocephalus* and *P. zeae*. The former species was also found

in cotton, crabgrass, peanuts, and corn but reproduced best in cotton and tobacco while the latter species was not found in cotton or peanuts. It was found in largest numbers in corn and crabgrass. However, Graham notes that the damage done to the hosts is not necessarily related to numbers. Thus he states that though maximum nematode populations in tobacco and corn were in a ratio 1:50, yet the tobacco roots were more injured than the corn. Tarjan (221, 224) and Steiner (214) have found meadow nematodes the primary cause of root proliferation, defoliation, and chlorosis of boxwood. Thomas (237) has found coffee seedlings severely injured by meadow nematodes in India, and Allen & Jensen (2) have found black walnut, grape, fig, citrus, olive, peach, raspberry, and boysenberry injured by *Pratylenchus vulnus* in California.

List of Species with Type Hosts

1. *P. pratensis* (de Man) Filipjev.—Meadow, England.
2. *P. obtusus* (Bastian) Goodey.—Wheat roots, England.
3. *P. gulosus* (Kühn) n. Comb.—*Clematis jackmanni*, Germany.
4. *P. coffeae* (Zimmermann) Goodey).—Coffee, Java.
5. *P. sacchari* (Soltwedel) Filipjev.—Sugar cane, Java.
6. *P. penetrans* (Cobb) N. Comb.—Violet, New York.
7. *P. brachyurus* (Godfrey) Goodey.— Pineapple, Hawaii.
8. *P. mahogoni* (Cobb) Filipjev.—Mahogony, Barbadoes.
9. *P. musicola* (Cobb) Filipjev.—Banana, West Indies.
10. *P. leiocephalus* Steiner.—*Arachis hypogeae*, Alabama.
11. *P. scribneri* Steiner.—*Solanum tuberosum*, Tennessee.
12. *P. neglectus* (Rensch) n. Comb.—*Weingartneria canscens*, Germany.
13. *P. vulnus* Allen & Jensen.—*Juglans hindsii*, California.
14. *P. zeae* Graham.—Corn, South Carolina.

The Family Criconematidae

Members of this family were not formerly considered as plant pathogenes though Cobb had many unpublished notes indicating such relationship. Most of these forms (except the Dolichodorinae) are minute, sedentary, external feeders on plant roots apt to escape notice either because of attached soil particles [Steiner (214)] or because they wash free from the roots [Chitwood (33)]. Neither Goodey (112) nor Filipjev & Stekhoven (90) mention the possibility of these forms being detrimental to plants. The first actual record of parasitism was that of Cobb (63), who found *Paratylenchus nanus* and *P. anceps* feeding on roots of grasses and *Umbellaria californica*, respectively. Previously de Man in 1880 had described *P. macrophallus* from moist earth, Holland, and other investigators have not as yet established the food of this nematode. Linford, Oliveira & Ishii (153) found *P. minutus* feeding on pineapple roots but did not consider it pathogenic. Steiner (214) described *P. elastichus* as a parasite of *Boehmeria nivea;* Thorne & Allen (247) have described *P. hamatus* as a parasite of fig roots.

Thorne (243) and Allen & Jensen (1) described *Cacopaurus pestis* and

C. epacris associated with decline in *Juglans regia* and *J. hindsii*, respectively. Quite a large number of species in the genera *Criconema*, *Criconemoides*, and *Hemicycliophora* have been described as associates of plant roots or feeding thereon. Menzel (171) considered *Criconemoides rusticum* the cause of chlorosis in grapevines while Jackson (127) associated the same species with deterioration of roots and little leaf disease of pine seedlings. The possibility of their pathogenicity is discussed by Steiner (214) and Chitwood (33). Apparently these organisms reproduce very slowly so that any injury caused by them would not appear as a result of ordinary inoculation procedures. When massive soil screening and sedimentation was used in the preparation of *Criconemoides simile* inoculum, chlorosis, stunting and leaf dropping on peach seedlings were quite obvious but one could not eliminate the possibility that some other deleterious organisms contributed to the manifestations.

Tylenchulus semipenetrans

The citrus nematode *Tylenchulus semipenetrans* has been of debated pathogenic significance since it was first described by Cobb (60) and the disease association discussed by Thomas (236). There has never been a question relative to the feeding of these organisms on the roots of citrus but their presence in rather large numbers on the roots of apparently healthy trees has made it extremely difficult to accept this species as a major pathogene. Citrus orchards are subject to various diseased conditions including "slow decline," "quick decline," "podre dumbre," and "tristeza." Gutierrez (121) has presented an extensive study on the life history and biology of the parasites in Argentina. The adult females bore part way into the rootlets, enlarge, and become sedentary. The greater part of their bodies remain external to the rootlet. Necrotic damage to the rootlet is apparent in the vicinity of the nematode's head. Only healthy roots are attacked. Parasites are difficult to find on decaying roots. Various fungi and other saprophytic organisms enter the diseased rootlets as secondary invaders.

Reports of the occurrence of this parasite are becoming synonymous with citrus plantings over the whole earth, though the parasites are apparently highly specific to citrus plants. Recent reports come from New South Wales by an anonymous author (5), Brazil by Carvalho (27), South Africa by Stofberg & Le Roux (219), Florida by Taylor (228), Uruguay by Bertelli & Bertelli (14), and Argentina by Marchionatto (166, 167, 168). Segregation of the actual damage done by this organism from other diseases has been most difficult. Stofberg & Le Roux (219) report considerable numbers of the nematode found on healthy trees and conclude greening of fruit is not caused by the nematode. Bertelli & Bertelli (14) admit the presence of nematodes on plants suffering from "podre dumbre de las raicillas" but after the application of liberal manure the plants recovered; they feel the condition in question is caused by a fungus. Marchionatto (166, 167, 168) investigating the same condition gives a good description of the leaf symptoms in slow de-

cline, which include chlorosis and small leaves. He found the disease most severe in sour orange, root decay being initiated by *T. semipenetrans*. Liberal manure and mulch ameliorated the disease. Godfrey (102) reported the citrus nematode from Texas for the first time, stating that it is apparently a recent introduction though "slow decline" has long been known in the citrus orchards of that state; hence he concluded the nematode is not the causal agent.

Bittencourt (16) and Fawcett (81) have both studied the disease known as "tristeza" of citrus. The former author found the nematode on the roots of diseased plants but did not think it the primary agent; the second author found "tristeza" to be caused by a virus, becoming manifest when grafted on sweet orange stock. Costa & Grant (64) have apparently completely eliminated the citrus nematode from consideration in "tristeza" by proving it is a virus transmitted by *Aphis citridis*.

California investigators have taken preliminary steps in establishing the true damage caused by the citrus nematodes. Foote & Gowans (91) found that fumigated soil after 40 years previous use as a citrus orchard grew lemon trees much more poorly than new land. White (262) found larvae in citrus soils three years after the old orchard had been uprooted. Baines (6, 7) added nematode larvae in large numbers to potted seedlings and found growth reduced by 12 per cent; population build up is slow, the optimum temperature being 77–88°F.

Rather extensive studies are being made of the host range and tolerance, resistance or immunity in various plants. Baines (7) and Baines *et al.* (8) have found *Poncirus trifoliata* highly resistant but not immune. *Balsamocitrus dawei, Clausena lansium, Murraya paniculata,* and *Severinia buxifolia* are immune. Baines & Thorne (10) reported the olive (Oleaceae) infected with the citrus nematode; this is the first case of infection of a plant outside the Rutaceae.

Other Root Parasites and Associates

Such nematodes as *Aphelenchus avenae* (sensu lato), *Aphelenchoides parietinus* (sensu lato) and *Panagrolaimus* species have long been associated with plant roots, often found within them, but proof of their pathogenicity has never been conclusive. Christie & Arndt (52) artificially infected cotton seedlings with each of these nematodes and produced pits on the roots through which fungi and other secondary organisms were able to attain a foothold. Since the nematodes were found internally, actually within cells of otherwise apparently healthy tissue, it seems reasonable to assume that they are facultative parasites. Attempts to prove these forms as a part of the disease complex known as "sore-shin" of cotton were unsuccessful. No further progress has been made in such investigations though similar organisms are found associated with root decay in most plants.

Cobb (62) first mentioned *Xiphinema americanum* as an external root parasite and subsequent workers have often found members of this genus

associated with roots, but no one has as yet been able to furnish concrete evidence of their association with decline nor have artificial inoculations proven their pathogenicity. Present opinion is that they are slow reproducing ectoparasitic plant feeders which would not cause disease manifestations unless present over an extremely long period. However, Christie & Perry (56) produced a condition they term "stubby root" in corn seedlings by inoculation with a related nematode *Trichodorus* species. These forms were observed feeding on the roots, particularly the root tips, which are often killed. Late stages of the disease exhibit considerable necrosis, probably caused by secondary invaders. Above ground symptoms include stunting and chlorosis. A wide range of cultivated plants were found to be affected by this parasite. Lownsbery & Tarjan (159) obtained significant growth differences in celery as a result of *Trichodorus* inoculum.

The genus *Dolichodorus* was first described by Cobb (60) from fresh water; Steiner (214) has found it parasitic on celery roots in Florida. According to Christie, it is probably a normal parasite of water chestnuts. Tarjan, Lownsbery & Hawley (226) produced significant decreases in growth of celery seedlings by artificial inoculation with this species.

Belonolaimus gracilis was described by Steiner (214) as an ectoparasite of *Pinus caribea*. Owens (187) has established the pathogenicity of this nematode to peanuts by artificial inoculations; it is a migratory feeder and causes minute lesions at each feeding point; theoretically a smaller number of such organisms would be required to cause disease than if they were sedentary. Symptoms caused on the plants include gnarled and stubby roots; the crop of both nuts and hay is reduced as a result. Other crops known to be affected by this pathogene include cotton, corn, soybeans, and strawberries.

The Genus Ditylenchus Filipjev

As in other groups, host range studies in the genus *Ditylenchus* have disclosed distinct heterogeneity among organisms commonly called stem and bulb eelworms. Unlike some of the other genera, all species of *Ditylenchus* do not parasitize a single part of the plant. The stem and bulb eelworms, clover eelworms, and rye eelworms are the best known but others parasitize plant roots or feed on fungi. With between 20 and 30 named species, the genus presents a difficult taxonomic problem. Recognition of the existence of "races" is quite old but these "races" have been inadequately studied from both morphological and host range standpoints. Mixed infections of two or more species undoubtedly contribute to the difficulties involved. Thorne (244) made a nice beginning by separating the teasel nematode, *Ditylenchus dipsaci* (Kühn) Filipjev from the potato rot nematode *Ditylenchus destructor* Thorne (244).

Horticulturists and pathologists have been making varietal tests in an attempt to develop resistance in many of our field crops. This work has led to the recognition of several races of "*Ditylenchus dipsaci*," which have not been identified taxonomically. Wilson (264) discusses the significance of

these races in various garden plants. Dallimore & Thorne (71) have identified the form attacking sugar beets as *D. destructor* and J. B. Goodey (110) found the same species in iris; Goodey & Goodey (117) found the same species in *Mentha arvensis* and *Sonchus arvensis*. Kotthoff (140, 141) differentiates infestation of potatoes, *D. destructor* attacking the tuber only while *D. dipsaci* attacks leaf, stem, and tuber; he further states that the rye race does not infect potatoes.

Various authors have characterized host ranges or varietal tests of red clover, white clover, lucerne, oat, rye, onion, and tobacco eelworms. Goodey (114) considered the oat and onion eelworms identical. Noble (182) reports transfer of strawberry eelworms to oats and beans. Goodey (115) found the oat eel did not attack clover and the red clover and rye eels were different. Fredericksen (95) distinguishes red clover, white clover, and lucerne eels giving host ranges which indicate partial transfers. It is quite obvious from these studies that plant breeders will need to take more pains in setting up critical means for selection of *Ditylenchus* resistant plants. Seinhorst (201) and Seinhorst & Dunlap (202) have worked out a standardized process whereby a known number of specimens of a given "race" are inserted into a growing plant. The necessity of such tests is indicated by the report of Smith (206) that the "resistant" alfalfa variety Nemastan is not resistant to eelworms from alfalfa in certain parts of this country. Identification of other specialized races such as that reported on tobacco by Kotte (139) is one of the major problems of taxonomy.

The existence of host varieties more or less resistant to particular "races" is reported by Nilsson-Leissner & Nilsson (181), Ragonese & Marco (190), Jenkin (129), Linnman (156), and Bingefors (15).

Other developments of interest in the genus *Ditylenchus* include the finding of *Ditylenchus radicicola* as a pathogen of importance to wheat in Canada by Vanterpool (261) and the finding of an unnamed species of *Ditylenchus* as the cause of "*Cephalothecium*" disease of mushrooms by Lambert *et al.* (143), and Cairns (19).

From the pathological standpoint, it is of interest to know that the bud leaf *Ditylenchus*, or those infecting narcissus, hyacinths, and strawberries, commonly form galls termed "spikkles." These are usually enlarged chlorotic areas of the leaf. The "spikkle" is formed as a result of the diffusion of nemic enzymes into the cells while they are as yet in a rather undifferentiated state. This causes enlargement of the individual cells, that is, cellular "hypertrophy" and certain changes in the nature of their cell inclusions. The injection of nematodes into fully formed leaves does not result in gall formation. A single nematode in the early stage of leaf development can cause the formation of a large sized gall. So it would seem advisable to produce artificially such galls by micro-injection of nemic enzyme extract at the proper stage of leaf development. Another interesting phase in *Ditylenchus* pathology is that occurring in scale and tuber tissues as in cases of onion bloat and potato rot. In such cases, the nematode enzymes apparently act primarily

upon the intercellular lamellae of fully differentiated storage cells. In so doing the cells are loosened so that a mealy condition results and as a consequence, after removal of the scale epidermis with slight shaking, nothing remains but a lace-like residue of vascular tissues. In this case also one would suppose that such symptoms might be produced by artificial injections by nemic enzymes. The common browning which occurs in the later stages of infection in tissues as bulbs and tubers is apparently an oxidation phenomenon. It has not yet been determined whether nemic enzymes are involved or only the peroxidases of the plant.

The Aphelenchs

The aphelenchs are now recognized as a superfamily which includes carnivorous species, ecto- and endo-parasites and associates of insects, fungus feeders and root feeders, and leaf and bud nematodes. The taxonomy and pathology of the group has shown little progress since 1941. Only the bud and leaf nematodes have been investigated sufficiently to warrant discussion. These organisms were monographed by Goffart (106), Goodey (111), and Junge (137). Christie (42, 44) differentiated *Aphelenchoides fragariae* as the cause of "spring dwarf" of strawberries, *A. besseyi* as the cause of "summer dwarf" of strawberries. Other recognized pathogens were *A. olesistus* of begonia and ferns, *A. ribes* of black currants, *A longicollis* of violets and pansies, and *A. aderholdi* of lily of the valley.

Courtney (65) has established *A. olesistus* as the disease agent in dieback of Croft Easter lilies. As in strawberry nematode diseases, the causal agent may not be present in the tissue or observable in large numbers when the symptoms of the disease are most pronounced, so diagnosis is often difficult.

Franklin (93) recently revised the nomenclature of several bud living aphelenchs as found in Britain. She points out that the organisms called *A. fragariae* by Goodey (111) do not agree with the description and illustration of that organism as given by Ritzema Bos. She identifies it as *A. ritzema-bosi* of chrysanthemums and cites evidence of host transfers in support of this contention. Cross transfer and morphologic similarity is submitted as evidence that *A. olesistus*, the fern eelworm, is a synonym of *A. fragariae*, and that *A. ribes*, the black currant eelworm, is a synonym of *A. ritzema-bosi*. The extremely wide host ranges of the bud and leaf eelworms has long posed the question of their specific validities. *A. ormerodis* is apparently considered a synonym of *A. parietinus* by Franklin, and she states that *A. besseyi* does not occur in strawberries in England. This is peculiar since the description of *A. besseyi* by Christie agrees remarkably with the illustration of *A. olesistus* by Stewart (218). We cannot as yet consider these matters as settled. Considering the extensive statistical studies made by Junge and the wide host transfers made by this author and others, a complete reassessment of aphelenchoids would appear to be in order. Discrepancies in morphologic descriptions indicate that something is seriously wrong. Resort to isolation of single

gravid females and the propagation of populations from these as Christie did in root-knot nematodes may provide a better basis for host range and morphologic study.

Thirumalachar (235) described a bud rot of areca palm caused by *Aphelenchoides* species.

Feder (82) has described a condition known as "yellow bud blight" in Vanda Miss Joachim orchids caused by *A. ritzema-bosi.* In this case the flower buds turn yellow, then brown and as much as 50 per cent blind flowers may occur. The life cycle of the parasite is completed in the bud and it apparently does not enter tissues as does the same species in chrysanthemum. However, the thermal death point (52°C.) and host range as well as morphology are identical with the chrysanthemum eelworm.

Yoshii & Yamamoto (267) described a new disease of Japanese rice seedlings caused by *Aphelenchoides oryzea* Yokoo. The leaves are shortened, yellow to white, the grain is poor, and the yield is reduced 10 to 30 per cent. This form lives ecto-parasitically in the young folded leaves and is spread on the inside of the glumes. In unhulled grain the nematodes survived three years storage. Transfers were made to Hokkaido and Italian millet proving that "ear blight" of these crops is due to the same organism. Cralley & Adair (69) report the same disease in Arkansas, where it was known as "white tip" disease. Symptoms are in general those of a deficiency.

Tenorio (234) has given an extensive description of red ring disease of coconuts caused by *Aphelenchoides cocophilus* and has worked out the insect carriers in Pernambuco.

The Genus Anguina Scopoli

The genus *Anguina* or grass, leaf, and seed nematodes is best known through the species *A. tritici* or wheat eelworm. This species is gradually being reported from all parts of the globe since it is carried in the diseased seed or gall. Control measures in the form of brine flotation of wheat seed, and more recently hot water treatment or even mechanical separation (see p. 173), appear to provide sound control measures when they are used. Interest in this subject is increased with evidence that the wheat eelworm acts as a carrier of other diseases. Cheo (28) found that if he inoculated soil with *Bacterium tritici* and planted, no infestation was obtained, but if *Anguina tritici* from *Bacterium tritici* infected wheat was added to the soil, a good infection occurred. Hypodermic inoculation of wheat seedlings was a failure. It would thus appear conclusive that *Anguina tritici* acts as a vector in this case. Leuckel (147) also reports *Dilophospora alopecuri* carried on the surface of *Anguina tritici.*

A related species *Anguina agrostis,* parasitic on bent grass, comes to notice primarily because the galls are poisonous to cattle, resulting in blind staggers and other symptoms of alkaloid poisoning, according to Hardison (123). Shaw & Muth (204) fed screenings of fescue, *Festuca rubra commutata,*

to cattle and sheep; nervous symptoms, incoordination, and abortion resulted. Internally hemorrhages of the large intestine, endocardium, and gall bladder were observed. Uninfected fescue produced no such symptoms. Thus the injury was clearly associated with nematode galls. However, filtered nematodes injected into the blood stream caused no injury.

PHYSIOLOGICAL BEHAVIOR AND CONTROL

Hatching Stimulants and Root Attractives

The existence of hatching stimulants or root attractives in sugar beet root diffusates was discovered by Baunacke (13) and Rensch (193). Investigation of such materials given off by plants has been confined primarily to root diffusates, hosts of nematodes of the genus *Heterodera*, though Linford (152) has demonstrated attractiveness of various plant tissues to members of the genera *Meloidogyne*, *Pratylenchus*, and *Rotylenchus*. Gadd & Loss (97) effectively demonstrated the existence of root attractives in the cases of *Pratylenchus pratensis* and root-knot nematode invasions of tea, *Crotalaria*, *Tephrosia*, *Desmodium*, and turnip. Differential effectiveness of sugar beet, potato, and other root diffusates as observed by Franklin (92), and the suggestion by Triffit (252) that such might be used to control the potato eelworm stimulated further investigation of such materials, particularly on potatoes.

Ellenby (80) took preliminary steps in the standardization of potato root diffusate for use in immunity trials.

Lownsbery (158) made an extended study of natural potato root diffusates primarily for the purpose of standardizing the assay of larval emigration from cysts. In the course of his investigations he demonstrated a 94 per cent reduction in larvae by the addition of a large volume of diffusates to soil.

Todd *et al.* (251) isolated the "potato eelworm hatching factor" from tomatoes, termed it eclepic acid, and considered it closely related to anhydrotetronic acid. British investigators, e.g., Calam *et al.* (20, 21), Marrian *et al.* (169), Russell *et al.* (195), have made great strides in the isolation and preparation of such materials. It is to be hoped that these investigators will eventually be able to produce synthetic hatching stimulants which may be used in attempted control of the pest.

Fenwick (84, 85) has analyzed data on larval emergence from cysts in potato root diffusate finding the best expression of the hatching curve as log time plus one. According to these investigations, cysts should be soaked 10 to 12 days before exposure, the tests should be conducted at 25°C., and tests should be extended such time as necessary to obtain evidence of the parameters of the hatching curve. The cumulative hatch is recorded until such time as the point of inflection of the hatching curve is passed. One cannot set an arbitrary time limit because of variances of cyst content behavior in materials with diverse histories.

Plant Nutrition and Mineral Content

Study of the mineral content of plants infected with nematodes was initiated by Vanha (259) and Wilfarth & Wimmer (263) in the case of diseased sugar beets. These workers found diseased beets characterized by lower calcium, phosphorus, magnesium, nitrogen, and a very low potassium content. Roemer (194) found that additional potassium increased the yield of diseased sugar beets. Shaw (203) described the chlorosis of sugar beet leaves associated with nematode infection.

Ellenby (77) tested various elements in an attempt to correct "potato sick" soils, finding that boric acid, zinc sulfate, and magnesium chloride gave increased yields. Of these substances 0.05 mg. of zinc per plant gave 40 per cent increase in tuber weight.

Parris & Jehle (188) reported root-knot infected Fordhook lima beans deficient in phosphorus despite an adequate supply of this substance in the soil. Tarjan (223) working with meadow nematode infected boxwoods found an increase in the calcium in leaves, stems, and roots; magnesium was higher in healthy stems, diseased leaves, and roots; sodium was higher in diseased stems and leaves; nitrogen and phosphorus were higher in healthy stems and leaves but higher in diseased roots (the increased amount of these elements in roots might be due to the nematode bodies); potassium was higher in healthy than in diseased stem, leaf, and root samples.

Chlorosis has long been a recognized symptom of deficiency diseases which are the result of various root parasitic nematodes. Chitwood, Specht & Havis (40) found changes in the Mg, Ca, and K concentrations of peach leaves on plants infected with various species of *Meloidogyne*. Concentrations in these plants appeared to be associated with immunological responses of the specific varieties.

Oteifa (186) tested the effect of potassium nutrition of the host plant on the reaction of Henderson Lima beans grown in sand culture to infection by a root-knot nematode, *Meloidogyne incognita*. He found that infected plants have a lower amount of nitrogen, phosphorus, calcium, and magnesium, and were very low in potassium as compared to control plants. He indicated that infected plants supplied with excessive amounts of potassium made almost normal growth and maintained mineral levels comparable with that of the controls. The same investigator (184) showed that the rate of oviposition of this nematode increased with the potassium supply. In view of his findings, it appears that nematode damage is correlated with the amount of potassium available to the host plant. Such correlations suggest that the root-knot nematodes may require a considerable amount of potassium for their development and thus may cause a marked injury when introduced to plants deficient in potassium.

This subject, which might well be called chemical pathology, is much too embryonic to permit generalizations. It appears obvious that root parasitic nematodes affect the mineral content and physiology of the host, but wheth-

er these effects are caused by reduction of absorptive surface, interference in translocation or metabolism, or by differential absorption of nutrient elements by the nematodes, cannot be stated at the present time. When we have learned something of the nutrients actually required by these parasites and the mode of action of their enzymes, it may be possible to better alleviate the deficiencies or to neutralize the nemic enzymes. Separation of mechanical damage, localized pathology, and systemic pathology will require considerable work.

Phytotherapy

Hot water treatments.—The use of hot water in the treatment of nematode infested plants has been common practice for more than 30 years. Most of the methods and principles were standardized before 1941. Basically one must investigate each possible treating stage of each host and each nematode species, determine relative lethal time temperatures, and make allowance for variability of heat penetration. Formalin has been used in treating baths to increase the efficacy of such treatment but some plants do not tolerate this material.

Chitwood, Haasis & Blanton (39) standardized the treatment of narcissus bulbs for *Ditylenchus* as 4 hr. in 0.5 per cent formalin at 110°F. Wood (266) found that storage at 80°F. tends to prevent bulb injury as a result of this treatment. Staniland (208) described standard treatment methods and tank construction; later the same author (209) suggests the use of chlorophenol at 0.12 to 0.5 per cent in the treating water. Miller, Courtney & Anderson (174) report on the stability of hot water formalin baths, finding very little reduction in formaldehyde concentration of water after as many as eight 4-hr. treatments of narcissus; any reduction in concentration is due primarily to dilution when live steam is used for heating purposes.

Courtney (66) and Gould (118) have developed a hot water formalin treatment of iris bulbs for *Ditylenchus*. This treatment consists of 3 hours in 0.5 per cent formalin at 110°F., particular attention being given to dates of digging and storage; damage was shown to depend on degree of development of the bulbs, five to six weeks storage being deemed proper.

Courtney & Breaky (67) reported successful treatment of Croft Easter lilies for *Aphelenchoides olesistus* as 1 hr. in 0.5 per cent formalin at 110°F.

Baines *et al.* (9) recommend the treatment of bare root citrus seedlings in hot water for 15 min. at 116°F. for the control of the citrus nematode, *Tylenchulus semipenetrans*. López (157) recommends the treatment of alfalfa seed in hot water for 10 min. at 55°C. for *Ditylenchus* control.

Since the establishment of a lethal-temperature-duration curve by Godfrey & Hoshino (103) for root-knot nematodes, no new facts have been uncovered relative to treatment principles for these organisms. Since the nematodes are internal, the time required to obtain adequate heat penetration for each plant product and the tolerance of the plant at its stage of development and activity must be worked out separately.

Burk & Tennyson (17) recommended a hot water treatment of 5 min. at 116°F. for sweet potatoes and Tilford (249) 30 min. at 122°F. for peonies. Similar work by Christie & Crossman (54) for *Aphelenchoides fragariae*, *A. ritzema-bosi*, and *A. besseyi* showed considerable differences between the species in this genus. Recommendable hot water treatments of strawberries, chrysanthemums, and ferns have not been worked out completely. Treatment of dormant chrysanthemum stools at 117°F. for 30 min. was successful in destroying the eelworms, but considerable damage was sustained in some varieties by this treatment. However, Mosley (175) describes an electrically heated tank for the treatment of chrysanthemum stools. With the newly developed parathion sprays, hot water treatment of chrysanthemums appears unnecessary. Cralley (68) recommends a 15-min. treatment of rice seed at 52–53°C. for the control of *Aphelenchoides cryzae*.

Treatment of wheat seed for *Anguina tritici* as recommended by Limber (151) consists of a 2-hr. presoak and 30-min. treatment in hot water at 122°F.

Triffit & Hurst (253) exposed *Heterodera rostochiensis* cysts to various temperatures and durations. This work was repeated by Chitwood (35) and on the basis of such work a treatment of tubers at 132°F. for 5 min. is considered satisfactory.

Chemotherapy.—The use of chemicals in the treatment of living plants against nematodes was attempted unsuccessfully by many workers prior to 1941. This is a new field, hardly begun, but one that promises much, particularly to growers of ornamentals and perennials.

Dimock (72) found that by adding 25 p.p.m. of sodium selenate to the soil, chrysanthemum eelworm disease caused by *Aphelenchoides ritzema-bosi* was completely suppressed. Fraser (94) has confirmed these findings. Tarjan (222, 223) found the addition of sodium selenate to the soil to be a means of reducing meadow nematode in boxwood roots, but he does not consider the treatment recommendable. Dimock & Ford (73) and Stapley (210) have found parathion sprays of chrysanthemum a highly successful means of combatting the chrysanthemum eelworm. They recommend spraying chrysanthemums with 0.25 to 0.50 lb. active wettable parathion per 100 gallons of water. Feder (82) successfully controlled chrysanthemum eelworms in Vanda Miss Joachim orchids by spraying with 2 to 4 lb. of 25 per cent wettable parathion per 100 gallons of water.

Sachs (196) found that he could control *Aphelenchoides olesistus* in ferns by painting the leaves four times daily with a 0.05 per cent solution of E605f for 37 days.

Goodey (113) disinfected onion seed carrying *Ditylenchus dipsaci* by fumigating at the rate of 30 ml. methyl bromide per liter of air space for 20 hr. The minimum lethal dose is based on concentration-time product of 600. Lubatti *et al.* (160) designed a chamber for the fumigation of 100 lb. onion seed and Lubatti & Smith (161) have worked out further details rela-

tive to fumigation procedures. This same fumigation process was found to be effective in disinfecting red clover and teasel seed by Goodey (109).

Other Control Measures

Byars (18) introduced brine flotation as a means of controlling *Anguina tritici* in wheat and the method has been considered very efficient. In fact, Chu (57) says that it completely eliminated the disease in materials he so handled. A 5-min. water flotation separation was found to reduce infected wheat grains from 2.3 to 0.13 per cent infected. However, Chu found any such method impractical under Chinese conditions. Therefore, with the aid of an engineer, he designed a "wheat nematode eliminator," which consists of a modified cylinder rotating 1° from horizontal and, depending on specific gravity differences, separates 99.7 to 99.95 per cent of the diseased from healthy seeds.

Christie (45) found that he could satisfactorily control chrysanthemum eelworm merely by taking only top cuttings from rapidly growing plants. It would appear that terminal buds may grow away from a nematode infection.

Leiper (146) reported the use of jet spray washing of potatoes for the control of *Heterodera rostochiensis*. Machmer (163) obtained 86 to 90 per cent reduction of cysts as a result of washing. Others have further developed this process.

Soil Treatment

Soil disinfestation by heat.—The use of steam or hot water in the treatment of soils for nematode infestation has been common practice for greenhouse and potting soils for nearly 50 years. Such practices have become standardized to the extent that they are agricultural engineering rather than nematological problems. Information on various modifications such as tile, rake, hot water drench, and electrical pasteurizing methods has been assembled in the form of bulletins and reviews by Newhall (179, 180), Dimock & Post (74), and Johnson (131). In greenhouses where there is an adequate supply of steam, thermal treatments are not very expensive. However, they do have certain defects in that organic matter is broken down and sometimes toxic products are formed which are not beneficial to plant growth. For such reasons growers are using thermal treatments less each year, preferring chemicals in the form of soil fumigants, However. in particular crops such as mushrooms, Cairns (19) showed that heating provides the best method available to date for killing nematodes.

Solids as soil fumigants or amendments.—Calcium cyanamide, sodium cyanide, cresylic acid, and other poisons had been tried for years as possible soil nematocides and though increased crops were often produced, the efficacy of the treatments, expense, or mechanical difficulties have long prevented such work from coming to fruition. Within the past 10 years such investigations have been conducted primarily in Britain. Ing & Small (126) re-

ported the use of calcium cyanamide at 2 lb. per sq. yard against *Heterodera rostochiensis;* Lammerts (144) found 0.5 per cent ethyl mercury iodide at 2 to 3 gm. per sq. foot effective against root-knot nematodes; Goffart (108) obtained growth benefits by using 100 to 500 gm. "Cystogon" per sq. meter; Speyer (207) used a 1:2400 solution of potassium iodide at 30 to 120 gallons per 100 sq. yards for control of root-knot nematodes; Ellenby (78, 79) reported allyl isothiocyanate as inhibitor of *Heterodera rostochiensis;* Jones (134) found calcium chloroacetate at 300 to 600 lb. per acre did not reduce the nematode population but increased sugar beet crops. In addition to the fumigant mentioned, various other solids are used to some extent for nematode control, principally in tobacco and other seed beds. The most popular of these is urea as indicated by Clayton *et al.* (58). Nitrites, particularly those of calcium and potassium, and sodium azide, have also been reported by the same workers (59) as giving good nematode control.

Liquid soil fumigants.—Formaldehyde was one of the first liquids used for soil treatment, because of its effectiveness against miscellaneous fungi. However, it is ineffective against nematodes in the soil unless in combination with heat. Carbon disulfide was introduced as a soil insecticide some 70 years ago. Attempts to control nematodes with this compound were subsequently made by many workers with erratic results caused chiefly by lack of an understanding of soil fumigation procedures. The compound is now considered a fairly good nematocide when used at a dose rate of 1000 to 2000 lb./acre. Matthews (170) discovered the nematocidal activity of chloropicrin, but it remained for Godfrey (99, 100) and Johnson & Godfrey (133) to put this information to practice. The development of methods of application and a basic understanding of soil fumigation required considerable testing, and the expense of chloropicrin brought about a search for less expensive compounds and development of better testing procedures. Such work was done by Taylor (227), Chitwood (29 to 32), Young (268), Taylor & McBeth (230, 231, 232), Gingrich & Haenseler (119). Development of spot treatment by Taylor & McBeth (231) was indicative of attempts to develop inexpensive field practices. However, soil fumigation continued to be a greenhouse and potting soil practice rather than a field practice, as indicated by Godfrey (101). Chemicals found more or less effective, in addition to carbon disulfide and chloropicrin, were ethylene dichloride and methyl bromide. Carter (25, 26) discovered and developed dichloropropane-dichloropropene mixture as a soil fumigant. This single discovery gave more impetus to the subject than all previous investigations for the reason that the material was relatively inexpensive and easy to handle. It also provided a means by which nematode-infested field soils could be treated and accurate estimates could be made of the damage to field crops caused by nematodes. Christie (46, 48) brought forth ethylene dibromide, allyl bromide, and ethylene chlorobromide as soil fumigants. Lear (145) introduced dichlorobutene, Kreutzer & Montagne (142) introduced chlorobromopropene. Christie (49), Thorne (241, 246), and Schmidt (199) investigated diffusion patterns in soil fumigation. McClellan,

Christie & Horn (162) made basic studies on soil temperature and moisture relations to nematocidal efficacy and Allen & Raski (3) made more concrete studies of the influence of soil type on the diffusion of soil fumigants. Articles presenting reports of field application of these fumigants have been so numerous in this country that a complete bibliography of the information could not be presented here. More recently, Taylor (229) reported the various chemicals used as nematocides and the methods of their application. Steiner (216) estimated that soil fumigation in the United States has now become a $5,000,000 a year industry. Experiment stations in many other parts of the world are testing the value of these fumigants.

Natural Enemies

It comes as somewhat of a surprise to most helminthologists that the literature on parasites and natural enemies of helminths is so vast as to warrant a book on the subject. Yet such a book has been published by Dollfus (75). Parasites and natural enemies of nematodes range from bacteria to mites with the protozoa, fungi, nematodes, and oligochaetes represented. Within the last decade, literature on nematode capturing fungi has predominated chiefly through the work of Deschiens, Descaqeaux, and Drechsler. Although the first nematode trapping fungus *Arthobothrys* was reported by Zopf (270) as feeding on the wheat eelworm, very little work was done on this group until the last two decades. Most of the publications have been strictly of a mycologic nature. Drechsler (76) lists 53 species known to feed on nematodes. Since his review some 20 additional papers have been published (See Helminthological Abstracts). These investigators have found that such fungi can be cultivated on oat straw and disseminated over pasture or field soil.

Such organisms undoubtedly contribute considerably to variance of field experiments dealing with phytoparasitic nematodes and may lead to many erroneous impressions. Thus the value of organic fertilizers has recently received quite a boost because of the fact that they often minimize damage caused by nematodes. Organic matter changes soil consistency and increases water-holding capacity but it also often acts as both inoculum and culture medium for various predatory fungi. Linford *et al.* (153 to 155) found additions of massive quantities of organic matter, such as chopped pineapple plants, gave consistently significant reduction in root galling of pineapple plants and increased their growth. In pot experiments with pineapple plants, Linford & Yap (154) provided artificial inoculum of root-knot nematodes and various nematode trapping fungi. One of the fungi, *Dactylella ellipsospora* was found to remain dominant over a period of 15 months, growth of the plants was clearly increased as was production of lateral roots, but the amount of galling was not greatly affected. One would suspect they were dealing with a surplus nematode population reduced just sufficiently by the fungi to prevent root mortality.

Oligochaetes constitute the most obvious dominant organism in decaying

vegetable matter and are commonly mistaken for nematodes by the layman and plant pathologist. They are often the one organism most easily seen in diseased plant tissues and for this reason there is a natural tendency to attribute the condition to them. So far as present evidence is available, oligochaetes do not feed on healthy plant tissues. They often constitute one of the members of disease complexes. To illustrate this point we may cite an example in which we were called into examine peanut plants with a slimy decay of the stems during the early wet part of the growing season. Examination revealed some sloughing of the cortical tissues, presence of oligochaetes, nematodes of the genera *Aphelenchoides*, *Seinura*, and *Panagrolaimus*, some protozoa, bacteria, and fungi. None of the organisms were known pathogenes. The local pathologist isolated several bacteria and fungi but was unable to produce the disease with them artificially. We suspected the *Aphelenchoides* species was feeding on the fungi, the *Panagrolaimus* feeding on bacteria, the *Seinura* feeding on *Aphenelchoides* and *Panagrolaimus*, and the oligochaetes appeared to be feeding on the entire complex. Such a situation can be embarrassing to a scientist, asked by farmers to diagnose, give prognosis and control of his sick plants. As a matter of caution we did not mention any of the organisms found but stated that the condition was somewhat like a "wet rot" resulting from the abnormally high rainfall. Later in the season dry weather came, the plants grew well with only a few brown scars on their stems, a good crop was produced and everyone was happy. It appeared to be a self-curing disease without a pathogenic organism. It is possible that many such complexes appear and disappear but it is difficult to prove the role of each of the many organisms involved. So far as we are aware only two workers have attempted to elucidate the place of oligochaetes in this system experimentally.

Jegen (128) found an inverse relation between the numbers of "*Tylenchus devastatrix*" and "*Aphelenchus*" in strawberry plants and the numbers of enchytraeids (*Enchytraeus*, *Pachydrilus*, *Fredericia*) in diseased plants, attributing this phenomenon to digestion of nematodes by enchytraeids. Jegen artificially infected diseased plants with enchytraeids and left others uninfected as controls; the control plants died while the infected plants recovered if the disease was not too far advanced. Schaerffenburg (198) investigated the relations of enchytraeids to *Heterodera schachtii* in sugar beets. Pots were inoculated with both sugar beet nematodes and enchytraeids of the genera *Fredericia* and *Enchytraeus*. Interestingly enough, he found that the disease was definitely arrested and plants recovered if the enchytraeids were added to the pots before the nematodes matured on the roots.

The possible use of enchytraeids as a means of biological control in combatting nematode diseases has not been explored sufficiently. If the sum total of our present knowledge is correct, their use for diseased ornamentals and perennials such as boxwood, orchard trees, and other high value plants would appear to be worthy of exploration.

Many protozoa and bacteria have been recorded as parasites of free-

living nematodes but there have been very few descriptions of such for the phytoparasites. Thorne (242) described a microsporidian *Dubosquia penetrans* occurring in the body of *Pratylenchus pratensis*.

Scattered observations of carnivorous activities of certain soil nematodes date from the time of Bastian and de Man, but their place in the natural control of plant parasites was first emphasized by Cobb (61). The predominant nematode carnivores of soils were found by him to be members of the families Mononchidae and Dorylaimidae. Steiner & Heinly (217) investigated the possibility of controlling phytoparasitic nematodes with *Mononchus papillatus*. They found that though these organisms are voracious feeders, their reproductive rate is relatively low. Thorne (238) went further into the study of mononchs, namely *Mylonchulus parabrachyurus* and *M. sigmaturus* as control factors, following their populations over a period of three years. Artificial inoculations of fields with mononchs have not proven a successful measure of phytoparasitic nematode control, primarily because of the slow reproductive rate of the carnivores.

Many members of the Dorylaimoidea have been recorded as carnivores by Thorne (240) and Thorne & Swanger (248), but like the mononchs their reproductive rate is usually rather low. However, one does encounter moderate populations of dorylaimids in the vicinity of plant roots and on occasion specimens have even been encountered inside plant roots feeding on phytoparasites. Under such circumstances they must be reckoned with as natural control agents.

Of the carnivorous soil nematodes, however, the predaceous aphelenchs would appear to offer both the most confusing and the most attractive possibilities as control agents. As a general rule the predaceous aphelenchs are long tailed, as described by Christie (43) and should probably be classified in the genus *Seinura*. They reproduce somewhat more rapidly than other predators and may not be as specific as to food requirements.

LITERATURE CITED

1. Allen, M. W., and Jensen, H. J., *Proc. Helminthol. Soc. Wash.*, **17**, 10–14 (1950)
2. Allen, M. W., and Jensen, H. J., *Proc. Helminthol. Soc. Wash.*, **18**, 47–52 (1951)
3. Allen, M. W., and Raski, D. J., *Phytopathology*, **40**, 1043–83 (1950)
4. Anderson, P. J., *Plant Disease Reptr.*, **31**, 21–23 (1947)
5. Anonymous, *Agr. Gaz. N. S. Wales*, **53**, 31–32 (1942)
6. Baines, R. C., *Calif. Agr.*, **4**, 8 (1950)
7. Baines, R. C., *Calif. Citrograph.*, **35**, 344–45 (1950)
8. Baines, R. C., Clarke, O. F., and Bitters, W. P., *Phytopathology*, **38**, 912 (1948)
9. Baines, R. C., Klotz, L. J., Clarke, O. F., and de Wolfe, T. E., *Phytopathology*, **39**, 858 (1949)
10. Baines, R. C., and Thorne, G., *Phytopathology*, **40**, 963 (1950)
11. Barrons, K. C., *J. Agr. Research*, **58**, 263–72 (1939)
12. Bastian, H. C., *Proc. Linnean Soc. Lond.*, **25**, 73–184 (1865)
13. Baunacke, W., *Arb. biol. Reichsantalt Land- u. Forstw. Berlin-Dahlem*, **2**, 185–288 (1922)
14. Bertelli, J. C., and Bertelli, L. K., *Rev. assoc. ing. agron. (Montevideo)*, **16**, 26–43 (1944)
15. Bingefors, S., *Svensk Frötidning*, **17**, 2–6 (1948)
16. Bittencourt, P. Y. C., *Biologica*, **10**, 47 (1944)
17. Burk, E. F., and Tennyson, G., *Proc. Am. Soc. Hort. Sci.*, **39**, 299–302 (1941)
18. Byars, L. P., *U. S. Dept. Agr. Bull., No. 1041* (1919)
19. Cairns, E. J., *Phytopathology*, **42**, 4 (1952)
20. Calam, C. T., Raistrick, H., and Todd, A. R., *Biochem. J.*, **45**, 513–19 (1949)
21. Calam, C. T., Raistrick, H., and Todd, A. R., *Biochem. J.*, **45**, 520–25 (1949)
22. Carroll, J., *J. Helminthol.*, **11**, 137–56 (1933)
23. Carroll, J., and McMahon, E., *J. Helminthol.*, **13**, 77–91 (1935)
24. Carroll, J., and McMahon, E., *J. Helminthol.*, **15**, 21–34 (1937)
25. Carter, W., *Science*, **97**, 383–84 (1943)
26. Carter, W., *J. Econ. Entomol.*, **38**, 35–44 (1945)
27. Carvalho, R. de S., *Rev. agr. Sao Paulo*, **17**, 423–34 (1942)
28. Cheo, C. C., *Ann. Applied Biol.*, **33**, 446–49 (1946)
29. Chitwood, B. G., *Proc. Helminthol. Soc. Wash.*, **5**, 68–75 (1938)
30. Chitwood, B. G., *Proc. Helminthol. Soc. Wash.*, **6**, 66–70 (1939)
31. Chitwood, B. G., *Florists Exchange and Hort. Trade World*, **95**, 9 (1940)
32. Chitwood, B. G., *Phytopathol.*, **31**, 818–24 (1941)
33. Chitwood, B. G., *Proc. Helminthol. Soc. Wash.*, **16**, 6–7 (1949)
34. Chitwood, B. G., *Proc. Helminthol. Soc. Wash.*, **16**, 90–104 (1949)
35. Chitwood, B. G., *U. S. Dept. Agr. Circ., No. 875*, 48 pp. (1951)
36. Chitwood, B. G., *Plant and Soil*, **3**, 47–50 (1951)
37. Chitwood, B. G., and Buhrer, E. M., *Proc. Helminthol. Soc. Wash.*, **13**, 54–56 (1946)
38. Chitwood, B. G., and Feldmesser, J., *Proc. Helminthol. Soc. Wash.*, **15**, 43–55 (1948)
39. Chitwood, B. G., Haasis, F. A., and Blanton, F. S., *Proc. Helminthol. Soc. Wash.*, **8**, 44–50 (1941)
40. Chitwood, B. G., Specht, A. W., and Havis, A. L., *Phytopathol.*, **41**, 559 (1951)
41. Christie, J. R., *Phytopathology*, **26**, 1–22 (1936)
42. Christie, J. R., *Phytopathology*, **28**, 587–91 (1938)

43. Christie, J. R., *J. Wash. Acad. Sci.*, **29**, 161–70 (1939)
44. Christie, J. R., *Proc. Helminthol. Soc. Wash.*, **9**, 82–84 (1942)
45. Christie, J. R., *Proc. Helminthol. Soc. Wash.*, **9**, 84–97 (1942)
46. Christie, J. R., *Proc. Helminthol. Soc. Wash.*, **12**, 14–19 (1945)
47. Christie, J. R., *Phytopathology*, **36**, 340–52 (1946)
48. Christie, J. R., *Proc. Helminthol. Soc. Wash.*, **14**, 23–28 (1947)
49. Christie, J. R., *Plant Disease Reptr.*, Suppl. 170, 21 pp. (1948)
50. Christie, J. R., *Proc. Helminthol. Soc. Wash.*, **16**, 104–8 (1949)
51. Christie, J. R., and Albin, F. E., *Proc. Helminthol. Soc. Wash.*, **11**, 27–31 (1944)
52. Christie, J. R., and Arndt, C. H., *Phytopathology*, **26**, 698–701 (1936)
53. Christie, J. R., and Cobb, G. S., *Proc. Helminthol. Soc. Wash.*, **8**, 23–26 (1941)
54. Christie, J. R., and Crossman, L., *Proc. Helminthol. Soc. Wash.*, **2**, 98–103 (1935)
55. Christie, J. R., and Havis, L., *Plant Disease Reptr.*, **32**, 510–14 (1948)
56. Christie, J. R., and Perry, V. G., *Science*, **113**, 491–93 (1951)
57. Chu, V. M., *Phytopathology*, **35**, 288–95 (1945)
58. Clayton, E. E., Gaines, J. G., Graham, T. W., and Todd, F. A., *Phytopathology*, **39**, 4 (1949)
59. Clayton, E. E., Gaines, J. G., Smith, T. E., and Graham, T. W., *Phytopathology*, **35**, 483 (1945)
60. Cobb, N. A., *J. Wash. Acad. Sci.*, **3**, 287–88 (1913)
61. Cobb, N. A., *J. Agr. Research*, **9**, 27–33 (1917)
62. Cobb, N. A., *U. S. Dept. Agr. Circ., No. 1*, 48 pp. (1918)
63. Cobb, N. A., *J. Wash. Acad. Sci.*, **13**, 254–57 (1923)
64. Costa, A. S., and Grant, T. J., *Phytopathology*, **41**, 105–13 (1951)
65. Courtney, W. D., *Phytopathology*, **35**, 572 (1945)
66. Courtney, W. D., *Proc. Bulb Growers Short Course, West. Wash. Exptl. Sta.*, 25–31 (1949)
67. Courtney, W. D., and Breaky, E. P., *Florists Rev.*, **96**, 37–38 (1945)
68. Cralley, E. M., *Phytopathology*, **39**, 5 (1949)
69. Cralley, E. M., and Adair, C. R., *Plant Disease Reptr.*, **33**, 257–59 (1949)
70. Currie, J. H., *Pacific Rural Press*, **133**, 628 (1937)
71. Dallimore, C. E., and Thorne, G., *Phytopathology*, **41**, 872–74 (1951)
72. Dimock, A. W., *Phytopathology*, **34**, 299 (1944)
73. Dimock, A. W., and Ford, C. H., *Phytopathology*, **40**, 7 (1950)
74. Dimock, A. W., and Post, K., *Cornell Univ., Agr. Expt. Sta. Mem., No. 635* (1944)
75. Dollfus, R. P., *Encyclopédic Biol.*, **26**, 1–481 (1946)
76. Drechsler, C., *Biol. Revs. Cambridge Phil. Soc.*, **16**, 285–90 (1941)
77. Ellenby, C., *Nature*, **149**, 50 (1942)
78. Ellenby, C., *Nature*, **155**, 544 (1945)
79. Ellenby, C., *Agriculture (Engl.)*, **53**, 219–23 (1946)
80. Ellenby, E., *Nature*, **154**, 363–64 (1944)
81. Fawcett, G. L., *Rev. ind. y agr. Tucumán*, **35**, 33–35 (1945)
82. Feder, W. A., *Phytopathology*, **41**, 938–39 (1951)
83. Feldmesser, J., and Fassuliotis, G., *J. Wash. Acad. Sci.*, **40**, 355–61 (1950)
84. Fenwick, D. W., *J. Helminthol.*, **13**, 157–70 (1949)
85. Fenwick, D. W., *J. Helminthol.*, **25**, 37–56 (1951)
86. Fenwick, D. W., and Franklin, M. T., *J. Helminthol.*, **20**, 67–114 (1942)
87. Fenwick, D. W., and Franklin, M. T., *J. Helminthol.*, **25**, 57–76 (1951)
88. Filipjev, I. N., *Smithsonian Misc. Collections, No. 89*, 1–63 (1934)

89. Filipjev, I. N., *Trans. Inst. Zool. Acad. Sci.* (*U.R.S.S.*), **3,** 537–50 (1936)
90. Filipjev, I. N., and Stekhoven, J. H., *A Manual of Agricultural Helminthology* (E. J. Brill, Leiden, Holland, 878 pp., 1941)
91. Foote, F. J., and Gowans, K. D., *Calif. Citrograph*, **32,** 522–23, 540–41 (1947)
92. Franklin, M. T., *J. Helminthol.*, **18,** 83–84 (1940)
93. Franklin, M. T., *Ann. Applied Biol.*, **37,** 1–8 (1950)
94. Fraser, L., *J. Australian Inst. Agr. Sci.*, **13,** 67–68 (1947)
95. Frederiksen, H., *Beretining om Landboforeningernes Virksomhed for Plantcarlen pac Sjaelland*, 245–79 (1950)
96. Gadd, C. H., *Tea Research Inst. Ceylon Bull., No. 12*, 131–39 (1939)
97. Gadd, C. H., and Loss, C. A., *Ann. Applied Biol.*, **28,** 39–51 (1941)
98. Godfrey, G. H., *J. Agr. Research*, **33,** 223–54 (1926)
99. Godfrey, G. H., *Pineapple News*, **2,** 202–3 (1928)
100. Godfrey, G. H., *Southern Seedsman*, **2,** 14; **3,** 20 (1939)
101. Godfrey, G. H., *Phytopathology*, **32,** 22 (1942)
102. Godfrey, G. H., *Plant Disease Reptr.*, **34,** 269–70 (1950)
103. Godfrey, G. H., and Hoshino, H. M., *J. Helminthol.*, **23,** 41–62 (1933)
104. Godfrey, G. H., and Oliveira, J., *Phytopathology*, **22,** 325–48 (1932)
105. Goffart, H., *Z. Parasitenk*, **2,** 97–120 (1929)
106. Goffart, H., *Pflanzenschutz*, **4,** 105 pp. (1930)
107. Goffart, H., *Arb. biol. Reichsanstalt Land- u. Fortw. Berlin-Dahlem*, **18,** 83–100 (1930)
108. Goffart, H., *Mitt. Biol. Reichsanstalt Land- u. Forstw.*, **64,** 62–67 (1941)
109. Goodey, J. B., *J. Helminthol.*, **23,** 171–74 (1949)
110. Goodey, J. B., *Ann. Applied Biol.*, **38,** 79–90 (1951)
111. Goodey, T., *J. Helminthol.*, **5,** 121–60 (1928)
112. Goodey, T., *Plant Parasitic Nematodes and the Diseases They Cause* (E. P. Dutton & Co., Inc., New York, N.Y., 306 pp., 1933)
113. Goodey, T., *J. Helminthol.*, **21,** 45–59 (1945)
114. Goodey, T., *J. Helminthol.*, **22,** 1–12 (1947)
115. Goodey, T., *Ann. Applied Biol.*, **37,** 324–27 (1950)
116. Goodey, T., *Soil and Fresh Water Nematodes* (Methuen & Co., Ltd., London, England, 390 pp., 1951)
117. Goodey, T., and Goodey, J. B., *J. Helminthol.*, **33,** 89–90 (1949)
118. Gould, C. J., *Wash. State College Eng. Expt. Sta. Series*, Bull. No. 424 (1950)
119. Gingrich, C. E., and Haenseler, C. M., *Proc. Helminthol. Soc. Wash.*, **8,** 50–53 (1941)
120. Graham, T. W., *Phytopathology*, **39,** 1–8 (1949)
121. Gutierrez, R. O., *Rev. Invest. Agricolas. Buenos Aires*, **1,** 119–46 (1947)
122. Hagemeyer, J. W., *Proc. Helminthol. Soc. Wash.*, **18,** 112–14 (1951)
123. Hardison, J. R., *Proc. Oregon Seed Growers League, 6th Meeting*, 69, **71,** 73–74 (1947)
124. Hastings, R. J., *Can. J. Research*, [D]**17,** 39–44 (1939)
125. Hastings, R. J., and Bosher, J. E., *Can. J. Research*, [C]**16,** 230–36 (1938)
126. Ing, E. G., and Small, T., *Rappart Dir. de la Ferme d'Expériences "Howard Davis"* (Trinité, Jersey, 1940)
127. Jackson, L. W. R., *Plant Disease Reptr.*, **32,** 192 (1948)
128. Jegen, H. G., *Vierteljahrsschr. naturforsch. Ges. Zürich*, **65,** 100–208 (1920)
129. Jenkin, T. J., *Ann. Applied Biol.*, **32,** 281 (1945)

130. Jenkins, W. A., *Phytopathology*, **38**, 528–41 (1948)
131. Johnson, J., *Soil Sci.*, **61**, 83–91 (1941)
132. Johnson, L. R., and Thompson, H. W., *Agriculture* (*Engl.*), **52**, 266–70 (1945)
133. Johnson, M. O., and Godfrey, G. H., *Ind. Eng. Chem.*, **24**, 311–13 (1932)
134. Jones, D. P., *Ann. Applied Biol.*, **34**, 240–45 (1947)
135. Jones, F. G. W., *Ann. Applied Biol.*, **37**, 407–40 (1950)
136. Jones, L. H., *J. Agr. Research*, **44**, 275–85 (1932)
137. Junge, W., *Z. Parasitenk.*, **10**, 559–607 (1938)
138. Kostoff, D., and Kendall, J., *Zentr. Bakt. Parasitenk.*, [II] **81**, 86–91 (1930)
139. Kotte, W., *Z. Pflanzenkrankh. Pflanzenschutz*, **53**, 37–42 (1943)
140. Kotthoff, P., *Z. Pflanzenkrankh. Pflanzenschutz*, **57**, 4–14 (1950)
141. Kotthoff, P., *Anz. Schädlingskunde*, **37**, 107–8 (1950)
142. Kreutzer, W. A., and Montagne, J. T. W., *Phytopathology*, **40**, 16 (1950)
143. Lambert, E. B., Steiner, G., and Drechsler, C., *Plant Disease Reptr.*, **33**, 252–53 (1949)
144. Lammerts, W. E., *Phytopathology*, **30**, 334–38 (1940)
145. Lear, B., *Phytopathology*, **40**, 17 (1950)
146. Leiper, R. T., *Imp. Bur. Agr. Parasitology*, No. 1 (1940)
147. Leuckel, R. W., *Plant Disease Reptr.*, **32**, 291–92 (1948)
148. Liebscher, G., *Deutsche Landw. Presse*, **17**, 436–37 (1890)
149. Liebscher, G., *Deutsche Landw. Presse*, **17**, 477 (1890)
150. Liebscher, G., *Deutsche Landw. Presse*, **17**, 672 (1890)
151. Limber, D. P., *Proc. Helminthol. Soc. Wash.*, **5**, 20–23 (1938)
152. Linford, M. B., *Proc. Helminthol. Soc. Wash.*, **6**, 11–18 (1939)
153. Linford, M. B., Oliveira, J. M., and Ishii, M., *Pacific Sci.*, **3**, 111–19 (1949)
154. Linford, M. B., and Yap, F., *Phytopathology*, **29**, 596–609 (1939)
155. Linford, M. B., and Oliveira, J. M., *Soil Sci.*, **45**, 127–40 (1938)
156. Linnman, N., *Lantmannen Svenskt Land*, **29**, 567–69 (1945)
157. López, C. V., *Publ. Inst. Sanidad Vegetal., Ministerio Agr. Buenos Aires*, [A]**2**, 36 pp. (1946)
158. Lownsbery, B. F., *Phytopathology*, **41**, 889–96 (1951)
159. Lownsbery, B. F., and Tarjan, A. C., *Plant Disease Reptr.*, **35**, 403–4 (1951)
160. Lubatti, O. F., Russel, J., and Parter, J. S., *J. Soc. Chem. Ind.* (*London*), **67**, 309–13 (1948)
161. Lubatti, O. F., and Smith, B., *J. Soc. Chem. Ind.* (*London*), **67**, 297–309 (1948)
162. McClellan, W. D., Christie, J. R., and Horn, N. L., *Phytopathology*, **39**, 272–83 (1949)
163. Machmer, J. H., *Phytopathology*, **36**, 686 (1946)
164. Malloch, W. S., *Phytopathology*, **13**, 436–50 (1923)
165. Man, J. G. de, *Die frei in der teinen Erde und im Süssen Wasser lebenden Nematoden der niederländischen Fauna* (E. J. Brill, Leiden, Holland, 206 pp., 1884)
166. Marchionatto, J. B., *Inst. Sanidad, Ministerio Agr. Buenos Aires*, [A]**1**, 15 pp. (1945)
167. Marchionatto, J. B., *Rev. argentina agron.*, **13**, 96–100 (1946)
168. Marchionatto, J. B., *Inst. de Sanidad Vegetal. Ministerio Agr. Buenos Aires*, [A]**3**, No. 35, 6 pp. (1947)
169. Marrian, D. H., Russell, P. B., Todd, A. R., and Waring, W. S., *Biochem. J.*, **45**, 524–28 (1949)
170. Matthews, D. J., *Ann. Rept. Cheshunt Expt. Research Sta.*, 18–21 (1919)

171. Menzel, R., *Anz. Schädlingskunde*, **17**, 117–20 (1941)
172. Micoletzky, H., *Arch. Naturgeschichte*, **87**, 1–650 (1922)
173. Miles, H. W., *J. Helminthol.*, **8**, 103–22 (1930)
174. Miller, V. L., Courtney, W. D., and Anderson, B., *Phytopathology*, **40**, 627–31 (1950)
175. Mosley, F. O., *Yearbook National Chrysanthemum Soc.*, 34–36 (1946)
176. Mortensen, R., and Kolpin, R., *Tids. Planteavl*, **15**, 145–58 (1908)
177. Nagakura, K., *Japan. J. Zool.*, **3**, 95–160 (1930)
178. Needham, J. T., *An Account of Some New Microscopical Discoveries* (Needham, F., London, England, 126 pp., 1745)
179. Newhall, A. G., *Agri. Exp. Sta. Ohio Bull., No. 451*, 1–60 (1930)
180. Newhall, A. G., and Chitwood, B. G., *Phytopathology*, **30**, 390–400 (1940)
181. Nilsson-Leissner, G., and Nilsson, F., *Imp. Agr. Bur. Publ., No. 3*, 15–51 (1940)
182. Noble, M., *Nature*, **164**, 31–32 (1949)
183. Oostenbrink, M., *Het aardappelaatje* (*Heterodera rostochiensis, Wollenweber*) *een geraalijke parasiet voor de eenzrjdige aardappelcultuur* (H. Veenman & Zonen, Wageningen, Netherlands, 230 pp., 1950)
184. Oteifa, B. A., *J. Wash. Acad. Sci.*, **41**, 393–95 (1951)
185. Oteifa, B. A., *Phytopathol.*, **42**, 15–16 (1952)
186. Oteifa, B. A., *Proc. Helminthol. Soc. Wash.* (In press)
187. Owens, J. V., *Phytopathol.*, **41**, 29 (1951)
188. Parris, G. K., and Jehle, R. A., *Plant Disease Reptr.*, **27**, 235 (1943)
189. Poole, R. F., and Schmidt, R., *Agr. Exp. Sta. N. C. Bull., No. 265* (1929)
190. Ragonese, A. E., and Marco, P. R., *Rev. Agr. Agronomy*, **10**, 378–84 (1943)
191. Raski, D. J., *Phytopathol.*, **40**, 135–52 (1950)
192. Raski, D. J., *Plant Disease Reptr.*, **36**, 5–7 (1952)
193. Rensch, B., *Min. D. Landw. Ges.*, **22**, 412–14 (1924)
194. Roemer, T., *Handbuch des Zuckerrüben Bauses* (P. Parey, Berlin, Germany, 366 pp., 1927)
195. Russell, P. B., Todd, A. R., and Waring, W. S., *Biochem. J.*, **45**, 530–32 (1949)
196. Sachs, H., *Anz. Schädlingskunde*, **23**, 117–18 (1950)
197. Sasser, J. N., *Phytopathology*, **42**, 17–18 (1952)
198. Schaerffenburg, B., *Z. Pflanzenkrankh. Pflanzenschutz*, **57**, 183–91 (1950)
199. Schmidt, C. T., *J. Econ. Entomol.*, **40**, 829–37 (1947)
200. Schmidt, O., *Wiss. Arch. Landw.*, [A]**3**, 420–64 (1930)
201. Seinhorst, J. W., *Tijdschr. Plantenziekten*, **51**, 39–52 (1945)
202. Seinhorst, J. W., and Dunlop, M. J., *Tijdschr. Plantenziekten*, **51**, 73–81 (1945)
203. Shaw, H. B., *U. S. Dept. Agr. Bull., No. 722* (1916)
204. Shaw, J. N., and Muth, O. H., *J. Am. Vet. Med. Assoc.*, **114**, 315–17 (1949)
205. Smith, A. L., *Phytopathology*, **30**, 710 (1940)
206. Smith, O. F., *Phytopathology*, **41**, 189–90 (1951)
207. Speyer, E. R., *Ann. Rept. Cheshunt Expt. Research Sta.*, 48–50 (1943)
208. Staniland, L. N., *Agriculture* (*Engl.*), **54**, 278–82 (1947)
209. Staniland, L. W., *Ann. Applied Biol.*, **37**, 11–18 (1950)
210. Stapley, J. H., *Grower, London*, **34**, 935 (1950)
211. Steiner, G., *Phytopathology*, **15**, 499–534 (1925)
212. Steiner, G., *J. Agr. Research*, **35**, 961–81 (1927)
213. Steiner, G., *Phytopathology*, **35**, 935–37 (1945)

214. Steiner, G., *Soil Sci. Soc. Florida Proc.* **4B,** 72–117 (1949)
215. Steiner, G., *Natl. Shade Tree Conf. Proc.*, **25,** 108–18 (1949)
216. Steiner, G., *Plant Disease Reptr. Suppl.*, **195,** 463–70 (1950)
217. Steiner, G., and Heinly, H., *J. Wash. Acad. Sci.*, **12,** 367–86 (1922)
218. Stewart, F. H., *Parasitology*, **13,** 160–79 (1921)
219. Stofberg, F. J., and LeRoux, J. E., *Farming in S. Africa*, **18,** 259–60, 280 (1943)
220. Strubell, A., *Zool. Anz.*, **19,** 42–46, 62–66 (1887)
221. Tarjan, A. C., *Phytopathology*, **38,** 377 (1948)
222. Tarjan, A. C., *Phytopathology*, **39,** 505 (1949)
223. Tarjan, A. C., *J. Wash. Acad. Sci.*, **40,** 157–60 (1950)
224. Tarjan, A. C., *Phytopathology*, **40,** 1111–24 (1950)
225. Tarjan, A. C., *Phytopathology*, **42,** 20 (1952)
226. Tarjan, A. C., Lownsbery, B. F., and Hawley, W. O., *Phytopathology* (In press)
227. Taylor, A. L., *Proc. Helminthol. Soc. Wash.*, **6,** 62–66 (1939)
228. Taylor, A. L., *Plant Disease Reptr.*, **28,** 320–22 (1944)
229. Taylor, A. L., *Advances in Agronomy*, **3,** 243–64 (1951)
230. Taylor, A. L., and McBeth, C. W., *Proc. Helminthol. Soc. Wash.*, **7,** 94–96 (1940)
231. Taylor, A. L., and McBeth, C. W., *Proc. Helminthol. Soc. Wash.*, **8,** 26–28 (1941)
232. Taylor, A. L., and McBeth, C. W., *Proc. Helminthol. Soc. Wash.*, **8,** 53–55 (1941)
233. Taylor, A. L., and Smith, A. L., *Plant Disease Reptr.*, Suppl. 124, 147–48 (1940)
234. Tenorio, R. P., *Bol. secretaria agr. ind. y. com. (Pernambuco)*, **15,** 204–21 (1948)
235. Thirumalachar, M. J., *Nature*, **157,** 106–7 (1946)
236. Thomas, E. E., *Agr. Exp. Sta. Calif. Circ., No. 85*, 1–14 (1913)
237. Thomas, K. M., *Monthly Bull. Indian Coffee Board, No. 12*, 5–6 (1948)
238. Thorne, G., *J. Agr. Research*, **34,** 265–85 (1927)
239. Thorne, G., *Sci. Agr.*, **8,** 707–10 (1928)
240. Thorne, G., *Proc. Helminthol. Soc. Wash.*, **4,** 2–16 (1937)
241. Thorne, G., *Proc. Helminthol. Soc. Wash.*, **6,** 60–62 (1939)
242. Thorne, G., *Proc. Helminthol. Soc. Wash.*, **7,** 51–53 (1940)
243. Thorne, G., *Proc. Helminthol. Soc. Wash.*, **10,** 78–83 (1943)
244. Thorne, G., *Proc. Helminthol. Soc. Wash.*, **12,** 27–34 (1945)
245. Thorne, G., *Proc. Helminthol. Soc. Wash.*, **16,** 37–73 (1949)
246. Thorne, G., *Proc. Helminthol. Soc. Wash.*, **18,** 18–24 (1951)
247. Thorne, G., and Allen, M. W., *Proc. Helminthol. Soc. Wash.*, **17,** 27–35 (1950)
248. Thorne, G., and Swanger, N. H., *Capita Zool.*, **6,** 1–223 (1936)
249. Tilford, P. E., *Agr. Expt. Sta. Ohio Bi-Monthly Bull., No. 25*, 132–34 (1940)
250. Tischler, G., *Ber. deut. botan. Ges.*, **20,** 95–107 (1902)
251. Todd, A. R., Calam, C. T., Marrian, D. H., *et al.*, *Food*, **16,** 274–75(1947) (Abstract)
252. Triffitt, M. J., *Imp. Bur. Agr. Parasitology Notes and Memoranda, No. 6* (1922)
253. Triffitt, M. J., and Hurst, R. H., *J. Helminthol.*, **13,** 219–22 (1935)
254. Tufts, W. P., *Proc. Am. Soc. Hort. Sci.*, **26,** 98–100 (1930)
255. Tufts, W. P., and Day, L. H., *Proc. Am. Soc. Hort. Sci.*, **31,** 75–82 (1934)
256. Tyler, J., *Hilgardia*, **7,** 373–88 (1933)
257. Tyler, J., *Hilgardia*, **7,** 391–415 (1933)
258. Tyler, J., *U. S. Dept. Agr. Misc. Pub., No. 406*, 91 pp. (1941)
259. Vanha, J., *Z. Zuckerind. Böhmen*, **17,** 157–82 (1893)
260. Valleau, W. D., and Johnson, E. M., *Phytopathology*, **37,** 838–41 (1947)

261. Vanterpool, T. C., *Sci. Agr.*, **28**, 200–5 (1948)
262. White, F. A., *Calif. Citrograph.*, **32**, 312–13 (1947)
263. Wilfarth, H., and Wimmer, G., *Z. Ver. deut. Zucker-Ind.*, **53**, 1–41 (1903)
264. Wilson, G. F., *J. Roy. Hort. Soc.*, **73**, 325–45 (1948)
265. Wollenweber, H. W., *Krankheiten u. Beschändigungen der Kartoffel. Forschungsinst. Kartoffelbau Arb.*, **7**, 56 pp. (1923)
266. Wood, J., *J. Roy. Hort. Soc.*, **69**, 298–304 (1944)
267. Yoshii, H., and Yamamoto, S., *J. Faculty Agr., Kyushu Univ.*, **9**, 209–33 (1950)
268. Young, P. A., *Phytopathology*, **30**, 711 (1940)
269. Zimmermann, H., *Arb. Forsch. Inst. Kartoffelbau, Berlin*, **8**, 152–54 (1927)
270. Zopf, W., *Nova Acad. Caes. Leop.-Carol.*, **52**, 313–41 (1888)

SOIL MICROBIOLOGY[1]

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A survey of the developments during the four years following the review by Smith (1) reveals certain trends in research, some of which have been briefly indicated by Gibson (2). While some of the trends represent comparatively new fields of interest, such as research on the rhizosphere, microbial antagonisms, and the relationship of microorganisms to soil texture, it must also be noted that nitrogen transformation, a topic that engaged the attention of the earliest soil bacteriologists, has remained a popular subject of investigation. In the space allotted a complete coverage of the literature is not feasible, and the choice of topics will be, therefore, to some degree a reflection of the interests of the reviewer.

The Soil Microorganisms

The bacterial flora.—Soil microbiological research has been directed for the most part towards a study of processes in which microorganisms are known to participate rather than towards an objective study of the microorganisms themselves. As a result of this emphasis on the biochemical, or functional, rather than the biological approach to soil bacteriology, relatively little is yet known of the organisms indigenous to soil. Many of these forms are similar in morphology to *Corynebacterium* and have been tentatively assigned to this genus. Conn & Dimmick (3), however, proposed a new genus, *Arthrobacter*, for the inclusion of *Bacterium globiforme* and related soil forms, as having characteristics sufficiently different from those of *Corynebacterium diphtheriae*, the type species, to warrant generic distinction. This was further advocated by Conn (4) in his review of the most abundant groups of soil bacteria. While admitting that accommodation of *B. globiforme* and related forms in a separate genus had much to recommend it, by avoiding undue extension of *Corynebacterium*, Lochhead (5) suggested the need for much wider surveys than have been so far reported before any satisfactory classification of members of this group, in view of their morphological irregularities, could be made. Furthermore, Clark & Carr (6) demonstrated motility and flagellation in cultures of various soil corynebacteria which had previously been known as nonmotile species, which finding provides further justification for placing such organisms in a genus distinct from *Corynebacterium*.

Though sporeforming bacteria represent but a small proportion of the bacteria in soil and are not regarded as part of the autochthonous microflora, they may be considered as soil types, normally quiescent but capable of increasing upon the addition of nitrogenous organic matter and participating in decomposition processes. Recent interest has centered in the capacity for

[1] The survey of the literature pertaining to this review was completed in December, 1951.

Contribution No. 343 from the Division of Bacteriology and Dairy Research.

antibiotic production shown by various species. However, since the work of Smith, Gordon & Clark (7), which did much to clarify the taxonomy of the aerobic sporeformers, little has been added to our understanding of the systematics of this group or of the role played in soils.

A few advances in technique for the microscopic examination of the soil microflora have been reported. Those described by Tchan (8) and Strugger (9) represent slight modifications of the older methods for examining stained suspensions or impressions of soil on slides and are of doubtful quantitative value, though the use of the fluorescent dye, acridinorange, as used by Strugger, is of interest in that it provides a differentiation of living bacteria, because of intravital absorption of the stain, from soil particles and dead organisms. Of the newer microscopic techniques, that devised by Jones & Mollison (10) is the most significant. Measured amounts of soil are suspended in a molten agar gel from which small drops are removed and allowed to solidify as thin films on a hemocytometer slide where solidification ensures the fixation of the soil constituents in their original distribution. The film, of precise dimensions, is floated onto a slide and finally stained after which the organisms may be counted. Data of statistical significance support the description of the method which provides probably the best so far devised for the enumeration of soil bacteria by microscopic technique.

Bacterial growth factors in soil.—Recent studies on the nutrition of soil bacteria have brought to light the wide diversity in requirements shown by various groups of the autochthonous microflora, many of which are dependent upon nutrilites present in soil. That vitamins of the B group do occur in soil is well known, and the pertinent literature up to 1950 has been reviewed by Schmidt (11). Roulet (12) and Roulet & Schopfer (13) identified thiamine, biotin, riboflavin, pyridoxine, nicotinic acid, *p*-aminobenzoic acid, and inositol in soil, relating their occurrence to the activity of microorganisms as well as to the presence of vitamin rich organic matter. Schmidt & Starkey (14) found much higher levels of riboflavin in the organic layers and litter of forest soils than in agricultural soil, the amounts decreasing with the decomposition processes. Pure riboflavin or pantothenic acid added to sterile soil disappeared rapidly upon inoculation with normal soil though not under sterile conditions, indicating that the disappearance was due to microbial action rather than to adsorption by soil colloids.

The presence of unidentified growth factors in soil was suggested by Lochhead & Chase (15) who showed the presence of a well defined group of bacteria dependent upon them. More recently Lochhead & Thexton (16, 17) found that for many of the organisms requiring growth factors supplied by soil extract the growth promoting effect of the latter could be replaced by vitamin B_{12}, and that various other soil bacteria with simple nutritional requirements were able to synthesize a factor, the nutrilite effect of which was related to the amount of vitamin B_{12} produced. The occurrence of this vitamin in soil may be ascribed at least partly to its synthesis by microorganisms. Robbins, Hervey & Stebbins (18) stated that as many as 50 per cent of the

organisms developing on plates seeded with soil produced vitamin B_{12}, while Burton & Lochhead (19) found 70 per cent of the bacteria from cultivated soils, as well as 84 per cent of those from uncultivated soils of Northern Canada, to be capable of synthesizing B_{12}-active substances. That many bacteria requiring soil extract show no response to known vitamins suggests the presence in soil of other as yet unidentified factors. Taylor (20) has recently ascribed the growth promoting effect of soil extract to the presence of calcium ions, replaceable by strontium and to some degree by magnesium.

Soil fungi.—Though a consideration of soil fungi as agents of plant disease lies beyond the scope of this review, fungi as normal soil inhabitants are properly of concern. Reference is made to the review by Chesters (21) of soil inhabiting fungi and to the surveys by Warcup (21a) and Garrett (22) of ecological groups of soil fungi. By relating the mode of development, or mycelial growth habit, to the type and distribution of the substrate within the soil complex, Garrett suggested a rational approach to the problem of grouping. Chesters (23) devised an ingenious though laborious method for isolating the dominant, actively growing fungi in soil at a given time, whereby tubes with capillary orifices and filled with suitable medium are immersed in soil. Species of fungi so isolated usually possess a widely spreading mycelium and grow actively in soil. A modification of the usual plating procedure for isolation was proposed by Warcup (24) using minute quantities of soil in place of normal suspensions to minimize dispersion of the soil in plating. The methods for isolating fungi have been critically reviewed by Garrett (22). To prevent bacterial growth on media used for the quantitative estimation of soil fungi without the use of acid, which precludes development of certain fungal species, Martin (25) and Martin & Harding (26) studied the effects of various bacterial growth inhibitors and found the addition of rose bengal and streptomycin effective in providing a suitable plating medium.

Protozoa.—Giant amoeboid organisms of the genus *Leptomyxa* were found by Singh (27) to be widely distributed in soils of Great Britain, their occurrence in unmanured soils indicating that they were normal soil inhabitants. Their development was not affected by pH changes over a wide range, provided suitable bacteria, notably nonchromogenic forms, were available as food. Singh (28) noted further that total numbers of active amoebae, in contrast to bacterial numbers, did not differ significantly between soils receiving mineral fertilizers and farmyard manure, and ascribed the different relative effects of the soil treatment on the two groups of organisms to changes in the nature of the bacterial flora with respect to forms serving as food for protozoa. The specificity of protozoa-bacteria relationships was further shown by Crump (29) who found excystment of one species of amoeba independent of the presence of bacteria, whereas a second species could not excyst without living bacteria of suitable type. Mention should also be made of the observations of Gray (30) who noted a free exchange of ciliates inhabiting inland waters and those occurring in the soil and concluded that no real distinction exists between such forms.

The Nitrifying Bacteria

The isolation of the nitrifying bacteria in pure culture is a difficult and tedious process, and attempts have been made to simplify the procedure. Meiklejohn (31) isolated the ammonia oxidizing organism, *Nitrosomonas europaea*, by use of a method involving a building up of a population of nitrifiers by frequent subculturing in a mineral medium, followed by removal by carbon dioxide of the cells adhering to the particles of chalk, and plating on silica gel. Isolation of the same organism in pure culture was reported by Jensen (32) who used a procedure involving alternate dilution and plating on ammonium sulfate agar. In view of the abnormally high oxygen demand of the nitrifiers, no doubt responsible for the difficulties in promoting preferential growth in static enrichment cultures, Lees (33) introduced a radical change in isolation methods by using a soil percolator containing glass beads and suitable ammonium sulphate medium fed with sterilized air, by means of which after subculturing to fresh percolating systems, nitrifiers were reported obtained in pure culture. The introduction of the perfusion technique by Lees & Quastel (34) in 1946, whereby an aerated liquid is made to percolate intermittently through a column of soil, has provided a valuable tool for the study of metabolic processes that has been applied particularly to research on the biochemistry of nitrification. This subject has been recently extensively reviewed by Quastel & Scholefield (35), consequently this discussion will be confined to aspects of the problem relating more especially to the activity of the nitrifiers as affected by soil conditions.

Chase (36) used the perfusion technique to compare the nitrifying capacities of field soils subjected to different cultural practices. Nitrification was greater under a mulch of legume hay than under a permanent sod, either with or without application of ammonium nitrate, and the absence of a lag phase in the case of the mulched soil suggested that with this treatment the population of nitrifying bacteria had been maintained at maximum level. Martin (37) advised caution in assuming that any proliferation of nitrifying bacteria in a perfusion unit is necessarily a reflection of their activity in the field. He found the nitrification rate in cultivated soil to be significantly lower than in grassland soil, concluding, however, that the perfusion technique reflects the nitrifying potential of soil rather than its field activity. In a study of nitrate formation as a measure of the availability of nitrogen in soil organic matter, Allison & Sterling (38) found that nitrate production from organic matter was directly correlated with total soil nitrogen in soils subjected to a wide variety of cropping and cultural treatments, and concluded that in a soil of given type and under like climatic conditions, humified organic matter is fairly uniform in quality regardless of past treatment. In prairie soils of low nitrate producing power, Halvorson & Caldwell (39) found stimulation of nitrification on application of sodium nitrate, a similar favorable effect resulting from the addition of moderate, though not of large, amounts of calcium carbonate. Goring & Clark (40) compared mineralization

of nitrogen and differences in microbial populations in cropped and fallow soil, and though significant differences were found for total numbers of bacteria, such were not observed for numbers of *Nitrosomonas* and *Nitrobacter*. Though more mineral nitrogen was found in the cropped soil after five weeks, less was evident after 13 weeks, even allowing for nitrogen removed by cropping. The nitrogen deficit in the cropped soils was ascribed to immobilization of the element, a point also emphasized by Bartholomew & Clark (41) and by Gerretsen (42), who found as much as 40 per cent more of the soluble nitrogen transformed into microbial proteins in planted as compared with unplanted soil. He further noted that active nitrification in light soils of buffering capacity sufficient to maintain a pH of 4.5 to 5.5 may result in serious loss of nitrogen through the accumulation of nitrous acid and its subsequent decomposition. Dhar & Pant (43) had previously noted considerable loss of nitrogen during the process of active nitrification of ammonium salts or other nitrogenous compounds. This loss was attributed to the simultaneous presence of ammonium and nitrite ions with consequent formation of ammonium nitrite and eventual liberation of free nitrogen. Under acid conditions loss may be partially due to the decomposition of nitrous acid. The influence of oxygen concentration was studied by Amer & Bartholomew (44) who concluded that the optimum concentration for nitrification in soil is about that contained in soil air.

Little has been noted in the years under review in support of the hypothesis that nitrification in soil, especially in tropical countries, is a photochemical rather than a bacteriological process; in fact, the more recent evidence tends to refute such a belief. Joshi & Biswas (45) conducted a series of carefully controlled experiments but found no evidence of the activity of sunlight in promoting nitrification, concluding that photo-nitrification, if it exists, can be of no practical importance.

Nonsymbiotic Nitrogen-Fixing Bacteria

Continued interest in the microbiological, biochemical, and agronomic aspects of nonsymbiotic nitrogen fixation has been manifest in recent years. Reference may be made to the review of the mechanism of biological nitrogen fixation by Virtanen (46) and to the survey by Jensen (47) of biological nitrogen fixation in relation to the world's nitrogen supply.

Azotobacter.—The world-wide distribution of *Azotobacter* is further attested in recent reports. Gonick & Reuszer (48) found *Azotobacter chroococcum* in 74 per cent, but *Azotobacter vinelandii* in only 0.7 per cent of 283 Colorado soils. Sixty per cent of 178 Polish soils studied by Maliszewska (49) contained *Azotobacter*, the organism occurring most abundantly in soils well provided with phosphate. Examining 151 Queensland soils, McKnight (50) found 77 per cent of the soils of pH 6.0 or higher to contain *Azotobacter*, whereas it occurred in but 14 per cent of the soils below pH 6.0. In soils with a water content below 10 per cent *Azotobacter* was detected much less frequently than in moister soils, and it occurred more often in cultivated than in

virgin soils, *A. chroococcum* being the only species encountered. In Danish soils Jensen (51) confirmed the correlation of the occurrence of *Azotobacter* with soil reaction, noting a tolerance limit of pH 5.8. The density of the organism also varied with the available phosphate content of the soil, though not with the humus content. Gorbunov (52) reported the generally abundant occurrence of *A. chroococcum* in soils of the Volga delta frequently subject to flooding, though numbers tended to decline with progressive leaching.

The importance of molybdenum in nitrogen fixation by *A. chroococcum* has been further stressed by Mulder (53), and Jensen (54) found it to be required by *A. indicum*, with maximum effect at 0.1 to 1.0 part per million. Jordan & Anderson (55) found boron, at concentrations up to seven parts per million, to increase nitrogen fixation by *A. chroococcum*. The effect of calcium on nitrogen fixation by *A. indicum* was studied by Jensen (54) who concluded that this element is neither essential nor stimulatory or else quite infinitesimal amounts are sufficient. Though the liming of acid soils to maintain a pH above 6.0 will usually transform them into a suitable habitat for *Azotobacter*, calcium in itself does not appear to be essential. Thus Gainey (56) found that addition of calcium did not make growth possible at the lower pH values, concluding that lack of available calcium was not responsible for the failure of *A. chroococcum* to grow below pH 5.9. Reporting on the effect of various nitrogenous substances, Fedorov (57) found nitrogen fixation to be reduced by inorganic nitrogen compounds, but not by amides, while some amino acids caused slight depression, others slight stimulation. Of various gases, hydrogen and nitrous oxide were found by Molnar, Burris & Wilson (58) to inhibit fixation, the effect of the latter being specific for fixation as distinct from assimilation of combined nitrogen by *Azotobacter*. The role of hydroxylamine as a possible intermediate has received further attention from Novak & Wilson (59) and Pethica, Roberts & Winter (60); both groups of workers agreed in suggesting that hydroxylamine is unlikely to be one of the stable intermediates. The relationship of weather conditions, chiefly barometric pressure, to nitrogen fixation by *Azotobacter*, which had been considered important by Bortels (61), was reexamined by Jensen (62). Periodic experiments over a 16 month period showed no correlation between nitrogen gains and meteorological changes, indicating that the conclusions of Bortels were not generally valid. The role of *Azotobacter* in the formation of humus is suggested by Wang (63) who compared the properties of the black substance produced by the action of the organism on benzoates, known to be present in soil, with those of humus; similarity between the two substances with respect to physical and chemical characteristics led to the conclusion that they are identical.

Morphologic and taxonomic study of *Azotobacter* has received relatively little attention. Pochon, Tchan & Wang (64) have confirmed the pronounced morphological variability characteristic of the genus. With the aid of phase and electron microscopy as well as nuclear staining techniques, Eisenstark *et al.* (65) observed four morphological cell types: large plump rods, small rods,

elongated and branched forms, and minute coccoid cells, in *Azotobacter agile* over a period of two months and suggested that the intracellular bodies found are similar to the nuclear bodies described for other bacteria. Of special taxonomic interest is the proposal by Derx (66) to establish a new genus, *Beijerinckia*. From acid forest soils of Java a nitrogen-fixing organism was isolated, identical with *A. indicum*, also isolated from tropical acid soil; however, in view of the differences in morphology, physiology, and cultural characteristics that distinguish the latter from other species of *Azotobacter*, Derx urges, with considerable justification, the setting up of a genus to include such forms with *A. indicum* as type species.

The use of cultures of *Azotobacter* to increase crop yields by inoculation of seed or soil adjacent to plant roots has continued to be a subject of investigation; however, reports of favorable results have, as previously, come chiefly from workers in Russia whose findings have not been substantiated elsewhere. Vozniakovskaia (67) reported improved yields of millet and wheat, particularly when, in addition to *Azotobacter*, *Clostridium pasteurianum* was included in the culture. According to Afanasieva & Voronova (68), wheat and oats responded to inoculation, the effectiveness of the treatment varying with the soil type, and being greatest on grey and dark grey podzol soils. Concomitant manurial treatments appeared to increase fixation. On the other hand, no clear-cut benefit could be observed with a wide variety of crops by other workers including Pochon, Chalaust & Tchan (69) in France, Fåhraeus *et al.* (70) in Sweden, Lesch (71), Schmidt (72) and Lehner (73) in Germany, Timonin (74) in Canada, Gainey (75) in the United States, and Stöckli (76) in Switzerland.

Clostridium.—Rosenblum & Wilson (77) showed that ability to fix nitrogen is more widespread among species of *Clostridium* than generally recognized. Use of the isotope N^{15} enabled recognition of the ability of 12 of 15 species tested to fix nitrogen. By improving technical details, the same workers (78) also showed that the efficiency of nitrogen fixation by *C. pasteurianum* could be increased two- to three-fold with its rate comparable with that of *Azotobacter*. The addition of molecular hydrogen to the atmosphere decreased the extent and efficiency of nitrogen fixation, though not the rate. In studying possible intermediates of fixation by *C. pasteurianum*, Rosenblum & Wilson (79) found only ammonia to be used at a rate and extent suggesting a true intermediate. Both hydroxylamine and hydrazine were toxic at low concentrations and no evidence was obtained of their being utilized even at nontoxic levels. Finally, Zelitch *et al.* (80), by recovering free ammonia with a high concentration of labeled nitrogen, demonstrated it to be a key intermediate in biological nitrogen fixation.

Other nitrogen-fixing organisms.—Though nonsymbiotic nitrogen fixation by bacteria has been generally considered to be carried out by *Azotobacter* and *Clostridium*, recent work has shown that this ability is by no means confined to these genera. Gest & Kamen (81) and Lindstrom, Burris & Wilson (82) demonstrated nitrogen fixation by the photosynthetic nonsulfur purple

bacterium, *Rhodospirillum rubrum*. By the further application of tracer techniques, Lindstrom *et al.* (83, 84) showed fixation by photosynthetic bacteria of the genera *Chromatium* and *Chlorobacterium*, representing purple and green sulfur bacteria respectively (83), as well as by other species of nonsulphur purple bacteria of the genera *Rhodopseudomonas* and *Rhodospirillum* (84). The ability of another group of bacteria to fix nitrogen was established by Sisler & ZoBell (85) who observed fixation by a species of autotrophic, anaerobic, sulfate-reducing bacteria of the genus *Desulfovibrio*. Of interest also is the report of a new nitrogen-fixing bacterium isolated by Gray & Smith (86); the organism, a long, motile, aerobic rod, appears to differ from other nitrogen fixers so far reported and has not been classified.

Though nitrogen fixation may be carried out by blue-green algae, this has received little emphasis. In rice soils, De & Sulaiman (87) found the growth of algae and nitrogen fixation to be increased in the presence of the crop, particularly if the soil was treated with nitrogen-free inorganic fertilizers; with the crop, fixation occurred even in soils of pH less than 6.0, in which normally no fixation took place. The stimulating effect of the crop was ascribed to an increased supply of carbon dioxide evolved as a result of respiration and root decomposition. The same authors (88) reported not only higher crop yields, but an increase in soil nitrogen in the presence of abundant algal growth. The beneficial effect of blue-green algae on the growth of rice plants was confirmed by Watanabe *et al.* (89) who found that in the presence of the alga *Tolypothrix tenuis* the nitrogen fixed amounted to approximately 20 lb. per acre.

Symbiotic Nitrogen-Fixing Bacteria

The fundamental as well as the more practical aspects of nitrogen fixation by bacteria living in symbiosis with leguminous plants continue to interest many investigators. The recent review of the biochemical and symbiotic properties of the rhizobia by Allen & Allen (90) has so adequately covered the literature that the present discussion will be restricted mainly to certain progress made since then. The reader is also referred to the brief, though valuable, summary by Thornton (91) of the relationship between nodule bacteria and the legume host plant and the problems presented by this association.

The mode of formation of the "swarmers," the small, spherical, motile elements occurring as a stage in the life cycle of *Rhizobium*, has been studied by Bisset & Hale (92) who ascribe their production to two independent processes. True swarmers were formed from the characteristic large, barred rods, being released by rupture of the cell wall; while with some strains, motile cells resembling swarmers were believed to arise by the normal process of cell division. To what extent these two methods are fixed characteristics is not clear, since strains of *Rhizobium* isolated from legumes considered to be infected by the same species showed different methods of swarmer formation. Though the use of serological methods has failed to re-

veal a clear-cut relationship between the nodule organisms and the host plants they infect, serology has become a useful tool for the identification of *Rhizobium* strains, their antigenic properties showing a high degree of stability, as shown by the work of Purchase, Vincent & Ward (93). Serological procedures were applied by Thornton & Kleczkowski (94) to identify nodules produced by field inoculation of clover. A determination of the percentage of strains, recovered from root nodules, positive to the antisera against selected inoculant strains, provided information of the behavior of these in infecting the crop and suggested a useful antigen tracer method. Though the decline or disappearance of nodule bacteria in soil and, in some cases, crop failure have been attributed to the action of *Rhizobium* bacteriophage, the importance of bacteriophage to legume crop production is still difficult to assess. Studying phage resistant mutants of *R. trifolii*, Kleczkowska (95) found phage resistance tending to coincide with mutations in other features, including effectiveness in nitrogen fixation. Ineffective phage resistant mutants were found to be much more readily developed from effective parent strains of *Rhizobium* than vice versa, suggesting that the deleterious effect of phage in soil may find expression in an increase in the proportion of ineffective strains.

The importance of the oxygen tension on symbiotic nitrogen fixation was shown by Bond (96) who found the growth of inoculated soya bean plants, as well as the weights of nodules per plant, though not the number of nodules, markedly reduced by curtailment of oxygen. Since the growth of uninoculated plants supplied with nitrate nitrogen was not affected at similar reduced oxygen levels, it was concluded that the effect on nodulated plants was exerted on the process of fixation or on associated processes of nodular respiration. Following the findings by Mulder (97) and Jensen (98) indicating that molybdenum is essential for symbiotic nitrogen fixation, Anderson & Spencer (99) reported that whereas only minute amounts of this element are required to permit nitrate utilization by the plants, appreciably greater quantities are needed in the symbiotic nitrogen fixation process. Though a deficiency of sulfur, as well as of molybdenum, inhibits nitrogen fixation, the evidence suggested that it affected nitrogen metabolism within the plant rather than the actual fixation process. Anderson & Spencer (100) further reported that whereas sulfur deficiency decreased the demand of the plant for nitrogen as well as the number of nodules formed, a deficiency of molybdenum had the opposite effect. That not all legumes respond to added molybdenum was shown by Mulder (101) who found a pronounced response by white and red clover on acid soils, though none by peas or beans. The possibility that species of *Rhizobium* capable of reducing nitrates to nitrites may contribute indirectly to losses of nitrogen through its liberation in gaseous form by interaction of nitrite with amino acids was re-explored by Allison & Doetsch (102); no indication of such loss was noted, and it was concluded unlikely that such does occur unless the pH falls below 5.0. Following the discovery by Virtanen *et al.* (103, 104) that a direct relationship exists between

the leghemoglobin content of nodules and the amount of nitrogen fixed, Jordan & Garrard (105) applied the technique to detect effective and ineffective rhizobia. Good agreement between leghemoglobin and yield was found, and in the case of a strain of *Rhizobium* suspected as being parasitic as distinct from ineffective, in that the inoculated plants suffered, the leghemoglobin values confirmed belief in such parasitism.

Various aspects of the infection of leguminous plants by *Rhizobium* and of the response to inoculation continue to be subjects of investigation; however, the factors controlling the invasion of roots by the bacteria and the effectiveness of fixation are so complex in their interaction as to restrict seriously a general application of findings obtained under a given set of conditions. Possibly the most significant of the more recent contributions has been that of Nutman (106, 107) who showed that in red clover successful nodulation of the plant is dependent upon genetic factors which determine the resistance or susceptibility to infection, thus demonstrating that the value of the symbiotic association is by no means simply a reflection of the character of the strain of *Rhizobium*. Baird (108) reported multiple infection of clover plants by strains of the nodule organism differing in nitrogen-fixing power. The desirability of using specially selected strains for different varieties of lespedeza is pointed out by Erdman (109); while Lynch & Sears (110) found the different strains of bacteria used to inoculate birdsfoot trefoil responded differently to the kinds and combinations of fertilizer materials, maximum effectiveness being dependent on an adequate supply of soil nutrients and favorable reaction. Nodulation of subterranean clover was found by Spencer (111) to be markedly improved by increased soil pH or increased calcium supply, although neither factor was effective alone. Increasing the amount of inoculum in the absence of lime resulted in increased nodulation. Bhaduri (112) correlated the numbers of rhizobia in the inoculum with the subsequent nodulation of *Phaseolus radiatus*. One week after inoculation, the percentage of infected root hairs was roughly proportional to the logarithm of the bacterial numbers supplied, the numbers of nodules at this stage already showing significant differences between extreme doses of inoculum; while after nine weeks final nodule numbers gave a better reflection of the amount of the original inoculum.

Cellulose Decomposition

Reference should be made to the extensive review by Janke (113) of the decomposition of cellulose by microorganisms, as well as to reviews of the anaerobic cellulolytic bacteria by Hungate (114) and McBee (115), dealing respectively with the mesophilic and thermophilic forms. The systematic position of *Cytophaga* was considered by Tchan, Pochon & Prévot (116) who proposed a modification of the generally accepted classification, the essential feature of which involved a subdivision of the family Cytophagaceae into three genera—*Sporocytophaga* Stanier, *Cytophaga* Winogradsky, and *Flexi-*

bacter Soriano. Clark (117) found that seven cellulolytic bacteria, heretofore assigned to *Cellulomonas* or *Bacterium*, had the morphological characteristics of *Corynebacterium;* however, whether such soil corynebacteria should be designated as species of *Corynebacterium*, or of *Arthrobacter*, as suggested by Conn & Dimmick (3), was considered a matter that should await better definition of the latter genus. The isolation of cellulose-fermenting bacteria in pure culture was found by McBee (118) to be facilitated by the use of cellobiose which provided a transparent solid medium in which contaminants are readily detectable, while Dickerman & Starr (119) introduced a modification of earlier procedures involving the use of cellulosic membranes formed by species of *Acetobacter*.

The symbiotic activities of cellulolytic bacteria and other forms have evoked some recent interest. Chalaust (120) noted more or less parallel variations in numbers of *Cytophaga* and *Azotobacter* in natural soils, as well as an increase in numbers of both organisms following enrichment of soil with cellulosic materials. Fåhreus (121) observed an increased decomposition of cellulose by *Cytophaga* when grown in association with *Agrobacterium radiobacter* which was attributed to the utilization by the latter of volatile acids formed in the course of the fermentation and harmful to the growth of *Cytophaga*. Notable stimulation of the cellulose-fermenting capacity of an obligate anaerobe when grown symbiotically with two noncellulolytic types was reported by Enebo (122). Cellulose decomposition in field soil has been studied by White *et al.* (123, 124) in relation to productivity and soil reaction. Significant correlations were found to exist between crop yields and the cellulose-decomposing power, as well as the nitrifying capacity of the soils (123), though not with the soil reaction (124).

Soil Microorganisms and Plant Roots

The rhizosphere.—The pronounced increase in numbers of microorganisms regularly noted in soil adjacent to plant roots, and particularly on root surfaces, over those in soil more distant from the plant, has directed interest to the relations existing between growing plants and the soil microflora. The microbiology of the rhizosphere has been discussed in recent reviews by Katznelson, Lochhead & Timonin (125) and Clark (126), and the reader is referred to these as well as to the review by Garrett (127), which though concerned primarily with the pathogenic root inhabiting fungi, thus extending outside of our present scope, is valuable in treating of interactions in a borderline field.

Timonin & Lochhead (128) found the density of bacteria, fungi, and algae in the rhizosphere of the tobacco plant to decrease with increasing distance from the base of the stem, this being particularly noticeable for cellulose-decomposing bacteria. In general, organisms with more complex nutritional needs were proportionately more numerous with increasing depth. Though sporeformers are not considered to be preferentially stimulated in the rhizo-

sphere, Clark and Smith (129) noted that certain species, e.g. *Bacillus circulans*, *B. brevis* and *B. polymyxa*, were relatively more numerous than others. That the rhizosphere effect depends upon living roots was shown by Vozniakovskaia (130) who found much larger numbers of microorganisms in the rhizosphere of living wheat roots than in that of dead roots, the latter being hardly different from control soil, though cellulose-decomposing types were stimulated by the dead roots. Denitrifying bacteria and *Clostridium* and *Achromobacter* forms were specially abundant in the rhizosphere of living roots. Daste (131) reported a multiplication of *Azotobacter* in the rhizosphere of wheat, followed, however, by a reduction suggestive of an inhibitory effect of root excretions. Though the juices of such plants as onion and garlic are known to possess antibacterial properties, these plants were found by Timonin & Thexton (132) to stimulate the development of microorganisms in soil adjacent to the roots, indicating that the active principle does not apparently diffuse sufficiently in soil to affect microbiological activity in the rhizosphere.

Further evidence has been adduced to show that different species or even varieties of plants may vary in their rhizosphere effect. Harper (133) noted that varieties of banana plants susceptible to Panama disease supported a larger rhizosphere microflora than resistant varieties and found the nutrient requirements of the microorganisms to be related to the sap of the plant variety in question. Comparing six crop plants, Wallace & Lochhead (134) found in all cases in the rhizosphere a much higher percentage of bacteria having simple nutritional requirements than in uncropped soil. However, significant differences were noted between certain crops in the proportions of bacteria of different nutritional requirements present in the rhizosphere, the differences existing, in the main, between legumes and grass crops. The value of a knowledge of the specific relationships between crops and soil microorganisms as an aid to a better understanding of the principles of crop sequence and related questions of plant nutrition and disease control was emphasized by these authors as well as by Krasilnikov (135) and Nilsson (136). It was pointed out earlier by Lochhead & Texton (137) that one of the characteristic rhizosphere effects is the preferential stimulation of bacteria requiring amino acids for maximum growth. Studying the more specific requirements of this group of organisms, Wallace & Lochhead (138) found the sulfur-containing amino acids, and especially methionine, to be the most significant. The same authors (139), from a comparative study of the morphology, physiological activity, and nutritional requirements of bacteria from seeds of various crop plants, concluded that the rhizosphere organisms could be regarded as a group intermediate between the indigenous soil bacteria and the epiphytic seed microflora.

Mycorrhiza.—The closely related subject of the mycorrhizal flora has been so adequately treated in recent reviews by Harley (140, 141) as well as by the same author (142) in this present volume that there is no need to attempt to cover the field here.

Microbial Antagonisms in Soil

The equilibrium between the various groups of organisms in soil at any given time doubtless depends in large measure upon the availability of appropriate nutrients, the supply of which in turn is influenced by many factors. However, much evidence has accumulated to support the belief that antagonisms between many microorganisms, which can be readily demonstrated *in vitro*, play a part in establishing the microbial balance in soils. The extension of interest in antibiosis to plant disease control leads beyond the scope of this paper, to a field recently reviewed by Weindling *et al.* (143); however, some reference to recent work emphasizing the microbiological aspects of the phenomenon of antibiosis in the soil appears in order, though a neat distinction is often difficult.

Antibiotics in soil.—Though many phenomena of microbial behavior in soil are most readily interpreted in terms of antibiotic action, there is surprisingly little direct evidence of antibiotic production in soil, a point further emphasized by Brian (144). What little is known of the fate of antibiotics added to soil indicates that they are inactivated at varying rates, usually more rapidly in nonsterile soil, pointing to some biological attack. Brian (144) found the persistence of gliotoxin to be mainly dependent on pH, the antibiotic being fairly persistent in acid soils. More recently Pramer & Starkey (145) reported that streptomycin, normally quite resistant to decomposition, is susceptible to attack by a species of *Pseudomonas*, while Siminoff & Gottlieb (146) found it to be effectively inactivated by soil colloids, and considered it improbable that basic antibiotics such as streptomycin play an active role in the soil. From soils cropped with legumes Casas-Campillo (147) obtained ether and alcohol soluble fractions exhibiting marked bacteriostatic action on *Rhizobium*. The fact that soils without legumes did not yield such substances suggested the establishment of *Rhizobium* as the factor responsible for their occurrence, which, considered in conjunction with their chemical characteristics, supported the hypothesis that the extracts were antibiotics. Of interest for its possible application is the work of Grossbard (148, 149) on the production of antibiotics, chiefly clavacin, in soil inoculated with appropriate fungi. In the presence of added carbohydrate, such as glucose, wheat straw, or sugar beet pulp, measurable amounts of antibiotics were produced in sterilized, as well as in partially sterilized soil, though not in natural soil. The difficulty of establishing a sufficiently active population to secure production of an antibiotic in normal soil has been stressed by Brian *et al.* (150).

Antagonistic microorganisms in soil.—Though the numerous studies of the antibiotic properties of organisms, mainly against animal and plant pathogens, that have been isolated from soil do not properly concern us here, the quantitative distribution of antagonistic types in soil is a matter of interest with respect to ecology and the microbiological equilibrium. Schatz & Hazen (151) found a higher percentage of antagonistic actinomycetes in forest soil than in field soil. Actinomycetes as a group contained a greater

proportion of active forms than fungi or bacteria, a finding also noted by Colligan (152) who observed a larger proportion of active forms at a depth of two to three inches than at the soil surface. In a survey of the actinomycete population of sugar-cane soils of Louisiana, Cooper & Chilton (153) found from 19 to 32 per cent of the isolates antagonistic to *Pythium arrhenomanes*, with the "antibiotic index," based on the degree of inhibition, varying with the soil reaction and reaching a maximum in soils of pH 7.5. Studying the effect of amendments to similar soils on the incidence of antibiotic forms, McGahan (154) observed a preferential stimulation of antibiotic actinomycetes following addition of cellulosic materials to soil, whereas nitrogenous materials favored the nonantibiotic forms. Soils of Northern Canada examined by Landerkin *et al.* (155) contained a high proportion of actinomycetes (61 per cent) that were active against one or more of eight test organisms. The proportion of antibiotic forms, as well as the antibiotic index, varied with the soil type but were greater in soil sampled at a depth of five to six inches than at zero to one or 10 to 11 inches. In comparing actinomycetes in soils of Argentina, Cercós & Rosemblit (156) found the southern soils of that country to be particularly rich in antibiotic types. The effect of growing plants on the distribution of antagonistic actinomycetes in soil was studied by Rouatt *et al.* (157) who found a greater percentage of actinomycetes active against four test organisms in the rhizosphere of four crop plants than in the control soils.

Our present knowledge, not only of the production and persistence of antibiotics in soil, but of the antagonisms prevailing between the members of the complex micropopulation, is still too fragmentary to permit any reliable assessment of the role of antibosis in affecting the microbiological equilibrium or to provide any rational basis for a system of modifying this balance. An illustration of the complexity of some of the issues involved was given by Lochhead & Landerkin (158) who demonstrated the existence of a wide range of cross-antagonisms among actinomycetes which, though all antagonistic to *Streptomyces scabies*, showed mutual antagonisms in varying degrees, making it doubtful just what influence might be exerted by a given type in a mixed population. Ciferri (159) and Thaysen (160), have expressed doubt as to the ability of organisms yielding antibiotic substances *in vitro* to produce such in soils or act as regulators of the microflora; however, Brian (144) points out that many observed facts can be explained on the assumption of antibiotic production and that the instability of antibiotics cannot be a factor precluding them from biological significance in soils.

Microorganisms and Soil Structure

The importance of soil structure in relation to moisture control and aeration has stimulated further study on the microbiological aspects of crumb formation, particularly water-stable aggregates. Gilmour *et al.* (161) found soil fungi capable of increasing the degree of aggregation in soils receiving additions of oat straw and more particularly, alfalfa; in the absence of the

fungi lesser increases in the unbound fraction were obtained. Comparing the aggregating power of various microorganisms in pure culture, Swaby (162) found fungi to exert the greatest binding effect, followed in order by actinomycetes, gum-forming bacteria, yeasts, and other bacteria, in general agreement with the results of McCalla (163). Swaby, however, cautions against interpreting field phenomena from results of pure culture experiments, showing that in a mixed population the effect may be weakened or nullified by antagonistic action, by the loosening of aggregates bound by fungal mycelia due to decomposition of the hyphae, and as shown in a later report (164), by disaggregating action on certain humus fractions. Swaby (164) noted improved aggregation following sterilization and reinoculation of soil, after which a decline was evident; however, van Bavel (165) found no significant effect from sterilization provided the treatment was followed by a wetting of the soil.

Bacterial polysaccharides of the levan and dextran types were found by Geoghegan & Brian (166) to exert a pronounced binding effect, producing stable aggregates that may, however, be eventually broken down by microbial action. Results suggested that although polysaccharides synthesized by microorganisms are not the sole agents concerned in aggregating soil, they may be of considerable importance. That bacteria capable of synthesizing polysaccharides, particularly those forming levans and glucose-uronic acids, are abundant in soils has been shown by Forsyth & Webley (167). Geoghegan (168) found dextrans synthesized by *Leuconostoc* species to be rather better aggregators than levans synthesized by *Bacillus subtilis*, while the glucose-uronic acid products of *Rhizobium* were much less effective. In the case of levans, the aggregating effect showed a significant correlation with their molecular weight. Molina & Spaini (169) also established that organic colloidal material formed in the course of the aerobic decomposition of cellulose has a pronounced effect in increasing the degree of water-stable aggregation in soil.

Availability of Plant Nutrients

Though fixation of plant nutrients by microorganisms may take place by conversion into microbial protoplasm during the course of active cell proliferation, changes in the availability of various inorganic elements may occur through oxidation or reduction processes with which microorganisms may be concerned. In soil deficient in available manganese Timonin & Giles (170) found that the application of calcium cyanide, but not of manganous sulphate, corrected deficiency symptoms in oats subsequently planted. The beneficial effects appeared to be correlated with a depression of cellulose-decomposing and, during the first months, of manganese-oxidizing organisms together with significantly higher levels of exchangeable manganese. Bromfield & Skerman (171) have suggested that the oxidation of manganese on Gerretsen's citrate medium, generally attributed to bacterial action, is a nonbiological process occurring in the presence of oxyacids at a suitably high

pH value resulting from microbial growth. Timonin (172) isolated fungi on Gerretsen's medium that were able to oxidize manganese in the absence of citrate and in both sterilized and unsterilized soil. Timonin (173) also reported different effects exerted on biological oxidation of manganese following the addition of various nitrogenous compounds to soil; whereas organic sources of nitrogen, such as urea or asparagine, enhanced oxidation, inorganic nitrogen retarded the process. In rice soils of pH 5.5 actinomycetes were regarded by Baars (174) as the active agents in the oxidation of manganese which occurs during the fallow and leads to deposits of manganic oxides.

The importance of microorganisms in making available to plants phosphorus present in insoluble form in soil was shown by Gerretsen (175). In the presence of the organisms the phosphorus intake by the plant was considerably greater than under sterile conditions where the intake was dependent alone on the solvent action of the roots. Pikovskaia (176) also found microorganisms an important factor in the mobilization of phosphorus in soil, even suggesting the inoculation of soil with active phosphate-decomposing bacteria. Kaila (177) studied the assimilation and mineralization of phosphorus by microorganisms in relation to the decomposition of organic materials, noting a relationship between the amount of phosphorus assimilated and the amount of organic matter broken down, and concluding that no mineralization of organic phosphorus occurs when the element is present in concentrations less than about 0.2 per cent.

Various Factors Influencing the Soil Microflora

Soil management.—Studying the effect of incorporating crop residues in soil, Dawson *et al.* (178) found greater numbers of microorganisms in the top soil when the residues were subtilled than when they were ploughed under, though differences were not apparent after nine months. Increased loosening of the soil, according to Mishustin & Shukowskaia (179), caused a stimulation of microbial activity, notably of nitrifying and cellulose-decomposing bacteria and *Azotobacter*. The prolonged use of inorganic fertilizers was reported by Mishustin & Prokoshev (180) to depress many bacterial groups, the beneficial effect of the application of manure in such cases being attributed in large measure to a stimulation of microbial activity. Mulder (181) found that the liming of an acid peat soil stimulated the development of actinomycetes more than that of bacteria and considered that in view of the ability of actinomycetes to attack less readily decomposable organic matter, they are chiefly responsible for the increase of available soil nitrogen following liming.

Herbicides, insecticides, and fungicides.—The effect on the soil microflora following use of the newer herbicides, insecticides, and fungicides has been a subject of recent interest, though differences in concentration and in methods of evaluating their effect make comparison difficult. Verona (182) found derivatives of 2,4-dichlorophenoxyacetic acid (2,4-D) applied at rates used in practice, to exert no significant effect on soil bacteria, though New-

man (183) and Jones (184) noted that the nitrifying organisms were among the most susceptible to more critical concentrations of the herbicide, confirming the work of Smith *et al.* (185). According to Jones (184) the inhibiting effect of 2,4-D on nitrification was increased in the presence of added nitrogen. It is generally agreed that any depressing effects on microbial activity in soil are of a transitory nature due to the inactivation of the chemical. That detoxication of 2,4-D in soil is due almost entirely to the activity of microorganisms was shown by Audus (186, 187) who found indications that the decomposition products may include a root growth stimulant. By an application of the perfusion technique, Audus found that three phases in the detoxication of 2,4-D and related compounds could be distinguished, involving an initial immediate adsorption onto soil colloids, followed by a lag or inactive phase and a final phase of rapid, complete detoxication following the proliferation of herbicide-decomposing organisms. An organism of the *Bacterium globiforme* group was found able to decompose 2,4-D in pure culture. That a microflora capable of decomposing 2,4-D is built up in the presence of the chemical is indicated by the work of Newman & Thomas (188) who found that pretreatment of the soil with 2,4-D or compounds having similar constituent groups resulted in a more rapid decomposition of 2,4-D subsequently applied. Since certain other related compounds had no effect on the persistence of 2,4-D, the microflora responsible for decomposition was considered highly specific. Reference should also be made to the brief review by Norman (189) on the factors affecting the persistence of the newer synthetic organic compounds in soil.

The newer insecticides and nematocides, provided they are applied at rates commonly used for pest control, do not appear to exert deleterious effects on the general microflora of the soil. Wilson & Choudhri (190) found little or no effect by benzene hexachloride on ammonification or on the numbers of bacteria, actinomycetes, and fungi in soil; however, some suppression of *Azotobacter* and soil algae was noted. On the other hand, Pochon & Lajudie (191) reported a slight stimulation of *Azotobacter*, as well as of nitrate-forming bacteria following application of benzene hexachloride and related compounds at concentrations customary in practice, though bacteria and fungi were in general unaffected. Stapp (192) likewise noted no depression of soil bacterial activity resulting from the use of two new phosphoric ester insecticides.

Halleck & Cochrane (193) studied the effect of various fungicides applied to the leaves of plants, but not directly to the soil, on the bacterial flora of the rhizosphere. Whereas certain compounds reduced the rhizosphere population as compared to the control soil, others had the effect of increasing the relative numbers of rhizosphere bacteria, the authors postulating that these agents caused some injury to the plant which resulted in an increased root excretion of compounds stimulating bacterial proliferation.

Soil sterilization.—In view of the changes in the properties of soil induced by the use of steam for sterilization, attention has been given to the applica-

tion of various volatile disinfectants for the purpose of sterilizing soil with minimum alteration to its physical and chemical character. Special interest has been centered on ethylene oxide which Dalton & Hurwitz (194) found superior to other disinfectants, both in its lethal qualities and in the lesser changes induced in soil. Clark (195) noted that the use of ethylene oxide greatly increased the total carbon content of soil, the added carbonaceous material being readily available for microorganisms. This increase in organic matter has been shown by Allison (196) to be the result of adsorption of ethylene oxide vapor and its hydrolysis to ethylene glycol. In spite of certain advantages, therefore, this fumigant is not capable of sterilizing soil without causing other important alterations.

LITERATURE CITED

1. Smith, N. R., *Ann. Rev. Microbiol.*, **2**, 453–84 (1948)
2. Gibson, T., *Agr. Progress*, **24** (2), 108–11 (1950)
3. Conn, H. J., and Dimmick, I., *J. Bact.*, **54**, 291–303 (1947)
4. Conn, H. J., *Bact. Revs.*, **12**, 257–73 (1948)
5. Lochhead, A. G., *J. Bact.*, **55**, 579–80 (1948)
6. Clark, F. E., and Carr, P. H., *J. Bact.*, **62**, 1–6 (1951)
7. Smith, N. R., Gordon, R. E., and Clark, F. E., *U. S. Dept. Agr. Misc. Pubs.*, **559**, 1–112 (1946)
8. Tchan, Y. T., *Ann. inst. Pasteur*, **73**, 695–96 (1947)
9. Strugger, S., *Can. J. Research*, [C] **26**, 188–93 (1948)
10. Jones, P. C. T., and Mollison, J. E., *J. Gen. Microbiol.*, **2**, 54–69 (1948)
11. Schmidt, E. L., *Soil. Sci.*, **71**, 129–40 (1951)
12. Roulet, M. A., *Experientia*, **4**, 149–50 (1948)
13. Roulet, M. A., and Schopfer, W. H., *Trans. 4th Intern. Congr. Soil Sci.*, **1**, 202–3 (1950)
14. Schmidt, E. L., and Starkey, R. L., *Soil Sci.*, **71**, 221–31 (1951)
15. Lochhead, A. G., and Chase, F. E., *Soil Sci.*, **55**, 185–95 (1943)
16. Lochhead, A. G., and Thexton, R. H., *Nature*, **167**, 1034 (1951)
17. Lochhead, A. G., and Thexton, R. H., *J. Bact.*, **63**, 219–26 (1952)
18. Robbins, W. J., Hervey, A., and Stebbins, E., *Am. J. Botany*, **37**, 370 (1950)
19. Burton, M. O., and Lochhead, A. G., *Can. J. Botany*, **29**, 352–59 (1951)
20. Taylor, C. B., *Nature*, **168**, 115–16 (1951)
21. Chesters, C. G. C., *Trans. Brit. Mycolog. Soc.*, **32**, 197–216 (1949)

21a. Warcup, J. H., *Trans. Brit. Mycolog. Soc.*, **34**, 376–99 (1951)

22. Garrett, S. D., *New Phytologist*, **50**, 149–66 (1951)
23. Chesters, C. G. C., *Trans. Brit. Mycolog. Soc.*, **30**, 100–17 (1948)
24. Warcup, J. H., *Nature*, **166**, 117–18 (1950)
25. Martin, J. P., *Soil Sci.*, **69**, 215–32 (1950)
26. Martin, J. P., and Harding, R. B., *Soil Sci. Soc. Am. Proc.*, **15**, 159–62 (1951)
27. Singh, B. N., *J. Gen. Microbiol.*, **2**, 8–14 (1948)
28. Singh, B. N., *J. Gen. Microbiol.*, **3**, 204–10 (1949)
29. Crump, L. M., *J. Gen. Microbiol.*, **4**, 16–21 (1950)
30. Gray, E., *Nature*, **161**, 854–55 (1948)
31. Meiklejohn, J., *J. Gen. Microbiol.*, **4**, 185–91 (1950)
32. Jensen, H. L., *Tid. Planteavl*, **54**, 62–80 (1950)

33. Lees, H., *Nature*, **167**, 355–56 (1951)
34. Lees, H., and Quastel, J. H., *Biochem. J.*, **40**, 803–15 (1946)
35. Quastel, J. H., and Scholefield, P. G., *Bact. Revs.*, **15**, 1–53 (1951)
36. Chase, F. E., *Sci. Agr.*, **28**, 315–20 (1948)
37. Martin, A. E., *J. Brit. Grassland Soc.*, **4**, 161–82 (1949)
38. Allison, F. E., and Sterling, L. D., *Soil Sci.*, **67**, 239–52 (1949)
39. Halvorson, A. R., and Caldwell, A. C., *Soil Sci. Soc. Am. Proc.*, **13**, 258–60 (1949)
40. Goring, C. A. I., and Clark, F. E., *Soil Sci. Soc. Am. Proc.*, **13**, 261–66 (1949)
41. Bartholomew, W. V., and Clark, F. E., *Trans. 4th Intern. Congr. Soil Sci.*, **2**, 112–13 (1950),
42. Gerretsen, F. C., *Trans. 4th Intern. Congr. Soil Sci.*, **2**, 114–17 (1950)
43. Dhar, N. R., and Pant, M. C., *J. Indian Chem. Soc.*, **26**, 227–30 (1949)
44. Amer, F. M., and Bartholomew, W. V., *Soil Sci.*, **71**, 215–19 (1951)
45. Joshi, N. V., and Biswas, S. C., *Indian J. Agr. Sci.*, **18**, 115–29 (1948)
46. Virtanen, A. I., *Ann. Rev. Microbiol.*, **2**, 485–506 (1948)
47. Jensen, H. L., *Trans. 4th Intern. Congr. Soil Sci.*, **1**, 165–72 (1950)
48. Gonick, W. N., and Reuszer, H. W., *Soil Sci. Soc. Am. Proc.*, **13**, 251–57 (1949)
49. Maliszewska, W., *Ann. Univ. Mariae Curie-Skłodowska*, [E]**4**, 37–56 (1949)
50. McKnight, T., *Queensland J. Agr. Sci.*, **6**, 177–94 (1949)
51. Jensen, H. L., *Tids. Planteavl*, **53**, 622–49 (1950)
52. Gorbunov, K. V., *Microbiology* (*U.S.S.R.*), **20**, 231–44 (1951)
53. Mulder, E. G., *Plant and Soil*, **1**, 94–119 (1948)
54. Jensen, H. L., *Proc. Linnean Soc. N. S. Wales*, **72**, 299–310 (1948)
55. Jordan, J. V., and Anderson, G. R., *Soil Sci.*, **69**, 311–19 (1950)
56. Gainey, P. L., *J. Agr. Research*, **76**, 265–70 (1948)
57. Fedorov, M. V., *Microbiology* (*U.S.S.R.*), **17**, 425–34 (1948)
58. Molnar, D. M., Burris, R. H., and Wilson, P. W., *J. Am. Chem. Soc.*, **70**, 1713–16 (1948)
59. Novak, R., and Wilson, P. W., *J. Bact.*, **55**, 517–24 (1948)
60. Pethica, B. A., Roberts, E. R., and Winter, E. R. S., *Nature*, **163**, 408 (1949)
61. Bortels, H., *Zentr. Bakt. Parasitenk.*, (II) **102**, 129–53 (1940)
62. Jensen, H. L., *Plant and Soil*, **2**, 301–10 (1950)
63. Wang, T. L., *Ann. agron.*, **1**, 316–32 (1950)
64. Pochon, J., Tchan, Y. T., and Wang, T. L., *Ann. inst. Pasteur*, **74**, 182–88 (1948)
65. Eisenstark, A., McMahon, K. J., and Eisenstark, R., *J. Bact.*, **59**, 75–81 (1950)
66. Derx, H. G., *Proc. Acad. Sci. Amsterdam*, **53**, 140–47 (1950)
67. Vozniakovskaia, Y. M., *Microbiology* (*U.S.S.R.*), **17**, 389–94 (1948)
68. Afanasieva, A. L., and Voronova, A. F., *Agrobiology* (*U.S.S.R.*), **1**, 100–7 (1950)
69. Pochon, J., Chalaust, R., and Tchan, Y. T., *Compt. rend. acad. agr. France*, **34**, 791–94 (1948)
70. Fåhraeus, G., Nilsson, R., Sundelin, G., and Rasmusson, J., *Lantbrukshögskol. Jordbruksförsöksanstalt. Medd.*, **24**, 1–55 (1948)
71. Lesch, W., *Z. Pflanzenernähr. Düngung u. Bodenk.*, **47**, 231–48 (1949)
72. Schmidt, O. C., *Z. Pflanzenernähr. Düngung u. Bodenk.*, **48**, 135–50 (1949)
73. Lehner, A., *Z. Pflanzenbau u. Pflanzenschutz*, **2**, 193–232 (1951)
74. Timonin, M. I., *Soil Sci. Soc. Am. Proc.*, **13**, 246–50 (1949)
75. Gainey, P. L., *J. Agr. Research*, **78**, 405–11 (1949)
76. Stöckli, A., *Landw. Jahrb. Schweiz*, **65**, 187–209 (1951)
77. Rosenblum, E. D., and Wilson, P. W., *J. Bact.*, **57**, 413–14 (1949)

78. Rosenblum, E. D., and Wilson, P. W., *J. Bact.*, **59**, 83–91 (1950)
79. Rosenblum, E. D., and Wilson, P. W., *J. Bact.*, **61**, 475–80 (1951)
80. Zelitch, I., Rosenblum, E. D., Burris, R. H., and Wilson, P. W., *J. Biol. Chem.*, **191**, 295–98 (1951)
81. Gest, H., and Kamen, M. D., *J. Bact.*, **58**, 239–45 (1949)
82. Lindstrom, E. S., Burris, R. H., and Wilson, P. W., *J. Bact.*, **58**, 313–16 (1949)
83. Lindstrom, E. S., Tove, S. R., and Wilson, P. W., *Science*, **112**, 197–98 (1950)
84. Lindstrom, E. S., Lewis, S. M., and Pinsky, M. J., *J. Bact.*, **61**, 481–87 (1951)
85. Sisler, F. D., and ZoBell, C. E., *Science*, **113**, 511–12 (1951)
86. Gray, E., and Smith, J. D., *J. Gen. Microbiol.*, **4**, 281–85 (1950)
87. De, P. K., and Sulaiman, M., *Soil Sci.*, **70**, 137–51 (1950)
88. De, P. K., and Sulaiman, M., *Indian J. Agr. Sci.*, **20**, 327–42 (1950)
89. Watanabe, A., Nishigaki, S., and Konishi, C., *Nature*, **168**, 748–49 (1951)
90. Allen, E. K., and Allen, O. N., *Bact. Revs.*, **14**, 273–330 (1950)
91. Thornton, H. G., *Agr. Progress*, **24** (2), 102–7 (1950)
92. Bisset, K. A., and Hale, C. M. F., *J. Gen. Microbiol.*, **5**, 592–95 (1951)
93. Purchase, H. F., Vincent, J. M., and Ward, L. M., *Proc. Linnean Soc. N. S. Wales*, **76**, 1–6 (1951)
94. Thornton, H. G., and Kleczkowski, J., *Nature*, **166**, 1118–19 (1950)
95. Kleczkowska, J., *J. Gen. Microbiol.*, **4**, 298–310 (1950)
96. Bond, G., *Ann. Botany*, **15**, 95–108 (1950)
97. Mulder, E. G., *Plant and Soil*, **1**, 94–119 (1948)
98. Jensen, H. L., *Proc. Linnean Soc. N. S. Wales*, **72**, 265–91 (1948)
99. Anderson, A. J., and Spencer, D., *Nature*, **164**, 273–74 (1949)
100. Anderson, A. J., and Spencer, D., *Australian J. Sci. Research*, [B] **3**, 431–49 (1950)
101. Mulder, E. G., *Trans. 4th Intern. Congr. Soil Sci.*, **2**, 124–27 (1950)
102. Allison, F. E., and Doetsch, J., *J. Bact.*, **61**, 47–50 (1951)
103. Virtanen, A. I., Jorma, J., Linkola, H., and Linnasalmi, A., *Acta Chem. Scand.*, **1**, 90–111 (1947)
104. Virtanen, A. I., Erkama, J. and Linkola, H., *Acta Chem. Scand.*, **1**, 861–70 (1947)
105. Jordan, D. C., and Garrard, E. H., *Can. J. Botany*, **29**, 360–72 (1951)
106. Nutman, P. S., *Nature*, **157**, 463–65 (1946)
107. Nutman, P. S., *Heredity*, **3**, 263–91 (1949)
108. Baird, K. J., *Nature*, **168**, 116–17 (1951)
109. Erdman, L. W., *Soil Sci. Soc. Am. Proc.*, **15**, 173–76 (1951)
110. Lynch, D. L., and Sears, O. H., *Soil Sci. Soc. Am. Proc.*, **15**, 176–80 (1951)
111. Spencer, D., *Australian J. Agr. Sci.*, **1**, 374–81 (1950)
112. Bhaduri, S. N., *Ann. Botany*, **15**, 209–17 (1951)
113. Janke, A., *Oesterr. botan. Z.*, **96**, 399–443 (1949)
114. Hungate, R. E., *Bact. Revs.*, **14**, 1–49 (1950)
115. McBee, R. H., *Bact. Revs.*, **14**, 51–63 (1950)
116. Tchan, Y. T., Pochon, J., and Prévot, A. R., *Ann. inst. Pasteur*, **74**, 394–400 (1948)
117. Clark, F. E., *Soil Sci. Soc. Am. Proc.*, **15**, 180–82 (1951)
118. McBee, R. H., *J. Bact.*, **56**, 653–63 (1948)
119. Dickerman, J. M., and Starr, T. J., *J. Bact.*, **62**, 133–34 (1951)
120. Chalaust, R., *Ann. inst. Pasteur*, **74**, 242–45 (1948)
121. Fåhraeus, G., *Ann. Royal Agr. Coll. Sweden*, **16**, 159–66 (1949)

122. Enebo, L., *Nature*, **163**, 805 (1949)
123. White, J. W., Holben, F. J., Jeffries, C. D., and Richer, A. C., *Soil Sci.*, **67**, 279–85 (1949)
124. White, J. W., Holben, F. J., and Jeffries, C. D., *Soil Sci.*, **68**, 229–35 (1949)
125. Katznelson, H., Lochhead, A. G., and Timonin, M. I., *Botan. Rev.*, **14**, 543–87 (1948)
126. Clark, F. E., *Advances in Agronomy*, **1**, 241–88 (1949)
127. Garrett, S. D., *Biol. Revs.*, **25**, 220–54 (1950)
128. Timonin, M. I., and Lochhead, A. G., *Trans. Royal Soc. Can.*, [V] **42**, 175–81 (1948)
129. Clark, F. E., and Smith, D. H., *Soil. Sci. Soc. Am. Proc.*, **14**, 199–202 (1950)
130. Vozniakovskaia, Y. M., *Microbiology* (*U.S.S.R.*), **17**, 458–62 (1948)
131. Daste, P., *Rev. gén. botan.*, **57**, 685–89 (1950)
132. Timonin, M. I., and Thexton, R. H., *Soil Sci. Soc. Am. Proc.*, **15**, 186–89 (1951)
133. Harper, J. L., *Plant and Soil*, **2**, 383–94 (1950)
134. Wallace, R. H., and Lochhead, A. G., *Soil Sci.*, **67**, 63–69 (1949)
135. Krasilnikov, N. A., *Microbiology* (*U.S.S.R.*), **18**, 49–58 (1949)
136. Nilsson, P. E., *Kgl. Lantbruksakad. Tid.*, **89**, 262–73 (1950)
137. Lochhead, A. G., and Thexton, R. H., *Can. J. Research*, [C] **25**, 20–26 (1947)
138. Wallace, R. H., and Lochhead, A. G., *Can. J. Research*, [C] **28**, 1–6 (1950)
139. Wallace, R. H., and Lochhead, A. G., *Soil Sci.*, **71**, 159–66 (1951)
140. Harley, J. L., *Biol. Revs.*, **23**, 127–58 (1948)
141. Harley, J. L., *New Phytologist*, **49**, 213–47 (1950)
142. Harley, J. L., *Ann. Rev. Microbiol.*, **6**, 367–86 (1952)
143. Weindling, R., Katznelson, H., and Beale, H. P., *Ann. Rev. Microbiol.*, **4**, 247–60 (1950)
144. Brian, P. W., *Symposia Soc. Exptl. Biol.*, **3**, 357–72 (1949)
145. Pramer, D., and Starkey, R. L., *Science*, **113**, 127 (1951)
146. Siminoff, P., and Gottlieb, D., *Phytopathology*, **41**, 420–30 (1951)
147. Casas-Campillo, C., *Anales. escuela nac. cienc. biol.* (*Mex.*), **4**, 339–52 (1947)
148. Grossbard, E., *Nature*, **161**, 614–15 (1948)
149. Grossbard, E., *Proc. 7th Intern. Botan. Congr.* (In press)
150. Brian, P. W., Wright, J. M., Stubbs, J., and Way, A. M., *Nature*, **167**, 347–49 (1951)
151. Schatz, A., and Hazen, E. L., *Mycologia*, **40**, 461–77 (1948)
152. Colligan, D., *Butler Univ. Botan. Studies*, **9**, 9–20 (1949)
153. Cooper, W. E., and Chilton, S. J. P., *Phytopathology*, **40**, 544–52 (1950)
154. McGahan, J. W., *Phytopathology*, **41**, 25 (1951)
155. Landerkin, G. B., Smith, J. R. G., and Lochhead, A. G., *Can. J. Research*, [C] **28**, 690–98 (1950)
156. Cercós, A. P., and Rosemblit, A., *Rev. argentina agron.*, **17**, 98–105 (1950)
157. Rouatt, J. W., Lechevalier, M., and Waksman, S. A., *Antibiotics and Chemother.*, **1**, 185–92 (1951)
158. Lochhead, A. G., and Landerkin, G. B., *Plant and Soil*, **1**, 271–76 (1949)
159. Ciferri, R., *Atti ist. botan. "Giovani Briosi" e lab. crittogam. Univ. Pavia*, **3**, 274–76 (1949)
160. Thaysen, A. C., *Nature*, **166**, 93–94 (1950)
161. Gilmour, C. M., Allen, O. N., and Truog, E., *Soil Sci. Soc. Am. Proc.*, **13**, 292–96 (1949)
162. Swaby, R. J., *J. Gen. Microbiol.*, **3**, 236–54 (1949)

163. McCalla, T. M., *Soil Sci. Soc. Am. Proc.*, **11**, 260–63 (1947)
164. Swaby, R. J., *J. Soil. Sci.*, **1**, 182–94 (1950)
165. Bavel, C. H. M. van, *Plant and Soil*, **2**, 395–404 (1950)
166. Geoghegan, M. J., and Brian, R. C., *Biochem. J.*, **43**, 5–13 (1948)
167. Forsyth, W. G. C., and Webley, D. M., *J. Gen. Microbiol.*, **3**, 395–99 (1949)
168. Geoghegan, M. J., *Trans. 4th Intern. Congr. Soil Sci.*, **1**, 198–201 (1950)
169. Molina, J. S., and Spaini, L. S., *Rev. argentina agron.*, **16**, 33–49 (1949)
170. Timonin, M. I., and Giles, G. R., *Proc. 7th Pacific Sci. Congr.* (In press)
171. Bromfield, S. M., and Skerman, V. B. D., *Soil Sci.*, **69**, 337–48 (1950)
172. Timonin, M. I., *Sci. Agr.*, **30**, 324–25 (1950)
173. Timonin, M. I., *Trans. 4th Intern, Congr. Soil Sci.*, **3**, 97–99 (1950)
174. Baars, J. K., *Trans. 4th Intern. Congr. Soil. Sci.*, **1**, 192–95 (1950)
175. Gerretsen, F. C., *Plant and Soil*, **1**, 51–81 (1948)
176. Pikovskaia, R. I., *Microbiology* (*U.S.S.R.*), **17**, 362–70 (1948)
177. Kaila, A., *Soil Sci.*, **68**, 279–89 (1949)
178. Dawson, R. C., Dawson, V. T., and McCalla, T. M., *Agr. Expt. Stas.* (*Nebraska*) *Research Bull.*, **155**, 3–26 (1948)
179. Mishustin, E. N., and Shukowskaia, P. N., *Z. Pflanzenernähr. Düngung u. Bodenk.*, **43**, 154–60 (1949)
180. Mishustin, E. N., and Prokoshev, V. N., *Microbiology* (*U.S.S.R.*), **18**, 30–41 (1949)
181. Mulder, E. G., *Trans. 4th Intern. Congr. Soil. Sci.*, **2**, 117–21 (1950)
182. Verona, O., *Ann. facoltà agrar. univ. Pisa*, **9**, 189–99 (1948)
183. Newman, A. S., *Soil Sci. Soc. Am. Proc.*, **12**, 217–21 (1948)
184. Jones, H. E., *J. Am. Soc. Agron.*, **40**, 522–26 (1948)
185. Smith, N. R., Dawson, V. T., and Wenzel, M. E., *Soil Sci. Soc. Am. Proc.*, **10**, 197–201 (1946)
186. Audus, L. J., *Plant and Soil*, **2**, 31–36 (1949)
187. Audus, L. J., *Plant and Soil*, **3**, 170–92 (1951)
188. Newman, A. S., and Thomas, J. R., *Soil Sci. Soc. Am. Proc.*, **14**, 160–64 (1950)
189. Norman, A. G., *Trans. 4th Intern. Congr. Soil. Sci.*, **3**, 100–2 (1950)
190. Wilson, J. K., and Choudhri, R. S., *J. Agr. Research*, **77**, 25–32 (1948)
191. Pochon, J., and Lajudie, J., *Compt. rend.*, **226**, 2091–92 (1948)
192. Stapp, C., *Nachrbl. deut. Pflanzenschutzdienst.*, **3**, 27–29 (1951)
193. Halleck, F. E., and Cochrane, V. W., *Phytopathology*, **40**, 715–18 (1950)
194. Dalton, F. H., and Hurwitz, C., *Soil Sci.*, **66**, 233–38 (1948)
195. Clark, F. E., *Soil Sci.*, **70**, 345–49 (1950)
196. Allison, L. E., *Soil Sci.*, **72**, 341–52 (1951)

THE CHARACTERIZATION OF ANTIBODIES[1]

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It is now firmly established that antibodies are modified serum globulins which can react specifically with antigenic substances, usually proteins or polysaccharides. This review will present some of the more recent data which permit characterization of antibodies in general and of differences among specific types of antibodies. In view of the broad scope of this subject, discussion will be largely limited to certain aspects of the physical and chemical characterization of antibodies and to immunochemical properties of antibody globulins. The earlier literature has been summarized by Kabat (1), Treffers (2), Campbell (3), Williams (4), and Mayer (5).

Physical and Chemical Properties and Purification

Electrophoresis.—Despite recognized limitations, the electrophoretic separation of the serum proteins offers the most convenient reference scheme for a consideration of the antibody-containing globulins. The conditions of analysis most generally used today are those suggested by Longsworth (6), namely, electrophoresis at pH 8.5 to 8.6 in a veronal (diethylbarbiturate) buffer of 0.1 ionic strength. This gives a separation into albumins, α-, β-, and γ-globulins. When several globulin peaks of each sort are present, they are designated as α_1, α_2, β_1, β_2, γ_1, γ_2 etc. in order of decreasing mobility.

Electrophoretic analysis of an animal serum before and after a course of immunization frequently demonstrates that the immune serum contains an increased amount of γ-globulin or of T-globulin, a fraction of intermediate mobility between the β and the more slowly migrating γ-globulins. This has been shown by the work of Tiselius & Kabat (7), and later by Kekwick & Record (8), van der Scheer *et al.* (9, 10), and by others. The T-globulin fraction may also be designated γ_1-globulin, in which case the more slowly migrating group of γ-globulins is called γ_2-globulins.

More direct evidence as to the antibody-containing fraction is afforded by comparing the electrophoretic pattern of immune serum with that of the same serum from which the antibodies have been absorbed by addition of specific antigen. This would be expected to give information only in those instances when the specific antibody globulin constitutes a substantial part of the particular globulin fraction. Tiselius & Kabat (7) found that absorption of immune serum of the rabbit and monkey produced a marked decrease in the γ-globulin content, whereas in horse antiserum to the pneumococcus,

[1] The survey of the literature pertaining to this review was concluded in December, 1951.

the decrease following absorption was in the T-globulin fraction. Similar observations were made later by many other investigators. Deutsch, Nichol & Cohn (11) noted a decrease in both α- and γ-globulins from the absorbed serum of chickens that had been immunized with human γ-globulin.

Another method of studying the properties of antibodies is by chemical or physical isolation of various globulin fractions from immune sera followed by determination of the relative concentrations of antibodies in the different fractions obtained. By dialysis against water, globulin can be separated into a water-soluble fraction (pseudoglobulin) and a water-insoluble fraction (euglobulin). Preparations obtained from serum by salting-out with sodium sulfate, ammonium sulfate, and potassium phosphate in the classical manner usually contain several electrophoretic components. More recently, fractionation of globulins of various animal species has been achieved with the use of low temperatures, controlled ionic strength and pH, organic solvent (usually ethanol), and a defined protein content. These methods were developed by Cohn and co-workers (12, 13). Modifications and extensions of this method to the separation of immune globulin fractions have been described by Oncley *et al.* (14) and by Deutsch and collaborators (15). Nichol & Deutsch (16) developed a procedure for the separation of γ_1-(or T) from γ_2-globulin, components with different mobilities but, as will be indicated later, fractions capable of further separation.

All γ-globulin fractions separated by chemical procedures show broad mobility ranges indicating electrical heterogeneity. Further subfractionation of γ-globulins is efficiently accomplished by the electrophoresis-convection apparatus of Kirkwood (17) as applied by Cann and co-workers (18 to 23). By this method bovine γ-globulin has been separated into four subfractions which possess different mobilities and isoelectric points. The isoelectric points for different γ (and T) globulin preparations are approximately between pH 6 and 7.4. As a group, they have the most alkaline isoelectric points of the plasma proteins, and, at neutral or weakly alkaline conditions, the slowest electrophoretic mobilities. However, some antibodies have isoelectric points as low as pH 4.5 (7).

The distribution of the antibodies in different globulin fractions should be mentioned. The antibody location in immune sera of the horse is complex. When horses are immunized by subcutaneous injections of certain toxoids or of rabbit serum albumin, egg albumin, hsiguasin, and hemocyanin (24), the resultant antibodies are concentrated in the water-soluble (pseudoglobulin) globulin fractions precipitated between 33 and 50 per cent saturation with ammonium sulfate. Electrophoretic studies of these immune sera frequently show an increase in the T- or γ_1-globulin fraction. When these antisera react *in vitro* with antigen, the reaction is characterized by a narrow zone of precipitation with formation of soluble complexes in both the zone of antigen excess and antibody excess. In contrast to these antibodies, horses immunized with repeated intravenous injections of bacteria, pneumococcal nucleoprotein, bacterial polysaccharides, and rabbit globulin (24, 25,

26) form antibodies which are found primarily in the water-insoluble (euglobulin) component of horse globulin. Electrophoretic studies of such immune sera frequently show an increase in the γ-globulin although an increase in T-globulin may also occur. Unlike the case of the toxin-antitoxin reaction, addition *in vitro* of antigen to an antiserum of this kind in the zone of antibody excess gives a measurable precipitate. These antibodies are often called bacterial, or polysaccharide, or precipitin-type antibodies as opposed to the antitoxin type of antibody, also referred to as flocculating antibody. These classical observations have been repeatedly confirmed, most recently, by Sandor and co-workers (27, 28), who find the antitoxins in the pseudoglobulin fraction and antibacterial antibodies associated with the euglobulins.

Alcohol precipitation methods have been employed for fractionation of equine hyperimmune sera. Smith & Gerlough (29) found in Fraction II (γ_2-globulin), 14,000 units of tetanus antitoxin per gm. of protein, in Fraction III (primarily T or γ_1-globulin), 5000 units of antitoxin per gm., and in Fraction IV, 11,700 units of antitoxin per gm. as assayed by guinea pig neutralization tests. Fraction IV, which contained mainly β-globulins, showed only 1 per cent γ_2-globulin and 8 per cent T-globulin on electrophoretic analysis. In this instance, the antitoxin was not confined to γ- and T-globulins. Deutsch & Nichol (30) investigated the distribution of antibodies in the sera of horses that had been immunized simultaneously with the toxoids of diphtheria, tetanus and *Clostridium perfringens* as well as with *Hemophilus pertussis*. The antitoxins appeared to be concentrated especially in the γ_1-globulin although considerable quantities were present in the γ_2-fraction. Very little of the antibody to *H. pertussis* was present in the γ_1-fraction, most of it being in the γ_2 component. The older observations of Tiselius & Kabat (7) on equine immune serum indicated that specifically precipitated antibody to the pneumococcal polysaccharide migrated as a T or γ_1-globulin.

Northrop & Goebel (33) have purified Type I pneumococcal antibody from horse serum by precipitation of the euglobulins by dilution with water. The precipitate was dissolved in saline and impurities removed by the addition of phthalate buffer. Electrophoresis of this fraction showed it to have the mobility of a γ-globulin which was nearly homogeneous. Further fractionation with ammonium sulfate gave several preparations of different solubilities which had similar antibody contents, 90 to 100 per cent precipitable with the polysaccharide, but with different protective values, agglutinating powers, and reactions with antihorse rabbit serum. The various fractions, including some which were poorly crystalline, did not behave as homogeneous preparations in solubility tests. Electrophoretic characterization of the various subfractions was not reported.

In the chicken, the study of Deutsch, Nichol & Cohn (11) affords indirect evidence that antibodies to human γ-globulin are in both α- and γ- globulin fractions. Hess & Deutsch (31) studied the distribution of antibodies in two cows that had been immunized with Newcastle virus and with *Brucella*

abortus. Neutralizing antibodies to the virus were found in both the γ_1- and γ_2-fractions. However, the antibodies responsible for hemagglutination inhibition to the virus were concentrated in the γ_1-fraction. Similarly, they showed a difference in distribution between agglutinins and bacteriocidins to *Br. abortus*.

Electrophoresis-convection was used by Cann, Campbell, Brown & Kirkwood (32) in a study of rabbit serum from animals immunized with a bovine globulin coupled with a *p*-azophenylarsenate dye. The antibody molecules were partially separated into fractions having different precipitabilities and different specificities, that is, as antiarsonillate and as antibovine globulin. The material with high antiarsonillate capacity appeared to be concentrated in the more slowly migrating γ-globulin fraction. Such electrophoretic differentiation of different antibodies in the same serum has also been described for human serum (see below).

In man, most of the antibodies are found in Fraction II of Cohn *et al.* (12), a fraction composed almost entirely of γ-globulin. Enders (34) studied the distribution of various antibodies in Fraction II and Fraction III-1 (mostly β-globulin) and found the antibodies to be greatly concentrated in Fraction II with the exception of the isoagglutinins and the typhi O antibody which were concentrated in Fraction III-1. Oncley *et al.* (14) determined the antibody content for Fractions II-1,2 and II-3 for a number of antigens, comparing the antibody content of a weighed amount of each fraction with that for an equal weight of plasma protein. Most of the antibodies appeared to be about equally distributed in Fractions II-1,2 and II-3. Again, it was shown that the isoagglutinins and typhi O antibodies in the human sera were concentrated primarily in Fraction III-1, only slightly more being present in II-3 than in II-1,2.

Cohn & Pappenheimer (35) demonstrated that the diphtheria antitoxin in human serum is associated primarily with the γ_2-fraction rather than γ_1-fraction as in horse serum. Hink & Johnson (36) found that in man, less than half of the total agglutinins to *H. pertussis* were in Fraction II, which contains most of the γ-globulin; however, almost all the mouse protective antibody to this organism was contained in the γ_2-fraction.

Using Method X of Cohn and his colleagues (13) for protein fractionation, Tauber *et al.* (37) demonstrated that the spirochetocidal activity of the serum of syphilitic patients is concentrated in Fraction III-1,2, a fraction also rich in isoagglutinins. Davis and co-workers (38) obtained Wassermann antibody from human serum by dissociation of floccules formed from human syphilitic serum with Kahn or Mazzini antigen. Dissociation of the complex was effected with 15 per cent sodium chloride or by extraction with alcohol and ether. In the ultracentrifuge the purified antibody had two components, one with an s_{20} of about 19 S and the other, an s_{20} of about 7 S. The Wassermann antibody was found to have an electrophoretic mobility intermediate between that of β- and γ-globulins in agreement with the studies

of Cooper (39), who found the antibody in β- or γ-fractions obtained by electrophoretic separation.

Using the electrophoresis-convection method, Cann *et al.* (40) fractionated a human Rh antiserum which contained both blocking antibodies and cryptagglutinoids. About 25 per cent of the latter were found to be associated with α- or β-globulins or both. The cryptagglutinoids appeared to be dispersed throughout the various γ-globulin fractions and the blocking antibody was found primarily in a γ-globulin fraction of low electrophoretic mobility.

Fractionation of two sera from allergic individuals was performed by electrophoresis-convection methods. Campbell, Cann, Friedman & Brown (41) report that the reagic activity, determined by Prausnitz-Küstner tests, was closely associated with α- and β-globulins.

The studies mentioned above indicate that certain antibodies in man and other species may be concentrated selectively, if not exclusively, in one or another subfractions of γ-globulin which differ in electrophoretic mobility, and occasionally may be found in fractions other than γ-globulin. As indirect evidence for the varying behavior of different types of antibodies in man, the work of Florman, Schick & Scalettar (42) is of considerable interest. It is well known that the antibody content to many antigens is approximately identical in maternal blood and cord blood (fetal) at the time of delivery. However, it is reported (42) that the titer for agglutinins to the streptococcus MG was four times greater for maternal than for fetal blood, and Hodes, Ziegeler & Zepp are quoted in a personal communication, as having found that antibodies to pneumococcal polysaccharides are considerably higher in maternal than in cord blood. Similar findings are reported by Adamson, Lõfgren & Malmnäs (43) who found H and O antibodies to *Escherichia coli* to be considerably higher in maternal than in cord blood. It may be noted that Longsworth, Curtis & Pembroke (44) had demonstrated earlier that γ-globulins of fetal sera were usually higher than those of the maternal sera as observed electrophoretically. In view of the predominant role that placental transmission of antibodies is assumed to play in the human, it is noteworthy that there is a large variation in the behavior of different antibodies.

The differential transmission of various antibodies through the human placenta is also observed in other species. Brambell and his colleagues (45, 46) have found that in the rabbit, antibodies are transmitted through the area vaculosa of the yolk-sac. In early development of the embryo, there is transmission of heterologous antibodies obtained from horse or ox as well as homologous ones; later, only homologous antibodies are transmitted. Hartley (47) has reported an interesting series of experiments with diphtheria antitoxin in which it was found that guinea pig antitoxin passed the placenta freely but that antitoxin digested with pepsin did not. Even undigested horse antitoxin was transmitted in small amounts through the placenta, but di-

gested antitoxin was not. Hartley also mentions cases of pregnant women with diphtheria; when treated with digested horse antitoxin, no antitoxin was found in the fetus.

Sternberger & Pressman (48) have described a new method for purification of antibody. Antigen is coupled with *p*-aminobenzenearsonic acid or with *o*-aminobenzoic acid by diazotization. This precipitates when added to antiserum to the original untreated antigen. The precipitate is dissolved in saturated calcium hydroxide solution and the antigen is removed from solution adsorbed to calcium aluminate, leaving the antibody in solution. In a subsequent report, Sternberger & Petermann (49) found that the antibody so treated was unaltered when the process was carried out at pH 11.4 but that some alteration of the antibody molecule occurred at pH 12.6. Previously, Kabat (50) had found that antibody is unstable at pH 12.4.

A novel method of antibody purification has been reported by Campbell, Luescher & Lerman (51) in which the antigen protein is coupled to powdered cellulose. This involves converting the cellulose to a *p*-nitrobenzyl derivative, followed by reduction of the nitro group, and subsequent diazotization and coupling in the usual way. With bovine albumin as the antigen, the cellulose-albumin complex was found to be an efficient specific adsorbent when used in a column to remove antibody from rabbit serum, although with some preparations of antigens not all the antibody was removed from the serum. Adsorbed antibody was eluted from the column with dilute acid. Earlier, Svensson (52) had purified antibody by first adsorbing antigen on charcoal, then adding antiserum, removing the excess protein by washing, and eluting the antibody from the adsorbent with dilute acetic acid.

Size of antibodies.—Pneumococcal antibodies studied by Kabat (50), which were isolated by specific precipitation with polysaccharides, gave two distinct sizes of molecules, those from cow, horse, and pig having a molecular weight of about 900,000 with a sedimentation constant of about 20 Svedberg units (s = 20 S), those from human, rabbit, and monkey serum about 160,000 to 180,000 with s = 7 S. Equine diphtheria antitoxin has been shown by Pappenheimer and co-workers (63) to have a molecular weight of about 180,000 (s = 7 S).

Studies in the ultracentrifuge by Williams *et al.* (53) and by Oncley, Scatchard & Brown (54) on γ-globulin fractions isolated by chemical procedures from human plasma gave about 75 per cent of material with 7 S and variable amounts of larger particles ranging from 8 to 18 S. Studies by Deutsch and co-workers (30, 31) and by Smith and Smith & Brown (55, 56) have also demonstrated variable amounts of heavy components in γ_1(T) and γ_2-fractions of high electrophoretic purity (95 to 99 per cent) obtained from human, bovine, and equine serum and from bovine colostrum and milk. The main components represented 80 to 95 per cent of the total and had sedimentation constants of about 7 S. In general, the amount of heavier material appeared to be greater in the fractions that had higher electrophoretic mobilities.

Although it had been suggested earlier (54, 55, 56) that the heavy components might be polymerically related to normal γ-globulins, further evidence for this has come from the study of Savat (57), who demonstrated that electrophoretically-separated γ-globulins contained almost entirely the components with 7 S. Recently, Cann *et al.* (58) have demonstrated that γ-globulins separated by electrophoresis-convection from individual human, bovine, and rabbit sera showed about 99 per cent of the 7 S component. They conclude that the larger components of γ-globulins are new components and are probably formed during ethanol fractionation procedures, although these investigators indicate that the possibility still remains that heavy components may be present in some individual sera or that they appear as a result of pooling samples from different individuals.

It may be noted, however, that the homogeneous heavy components with 20 S found by Kabat (50) were obtained by specific precipitation with antigen and that heavy components have also been observed in preparations from milk and colostrum isolated by salting-out methods with ammonium sulfate (55, 56). Pedersen (59) has also reported the presence of 20 S components in bovine and human sera; these were particularly high in human sera rich in isoagglutinins. Although the results of Savat (57) and Cann and collaborators (58) certainly suggest that chemical procedures of some sort produce the heavy components, further studies are needed. It would be desirable to compare the pneumococcal antibodies from horse antiserum isolated by electrophoresis-convection and those isolated by specific precipitation which have previously shown a single component with 20 S.

Composition.—Analyses of human γ-globulin (Fraction II of the Harvard group) have been reported by Brand *et al.* (60) and, for similar human γ-globulin fractions as well as for bovine and equine fractions, by Smith and coworkers (61, 62). Immune globulin fractions isolated from bovine colostrum and milk also have been analyzed; these resemble the serum proteins although minor differences are apparent (61, 62).

The immune proteins are all closely related as shown by a similar amino acid composition (Table I). These proteins represent as distinct a group as the serum albumins or hemoglobins of various species. Nevertheless, there are significant differences among various species and between fractions of different mobility obtained from the same species, although fractions of the same electrophoretic mobility from normal and hyperimmune animals appear not to differ.

Purified preparations of diphtheria antitoxin were shown to contain hexose (63). In later studies by Smith, Greene & Bartner (61) it was found that various immune globulin preparations contained about 2 to 3 per cent hexose and 1 to 1.5 per cent hexosamine as determined by colorimetric methods (Table I).

Table I presents a summary of the available information concerning the composition of immune protein fractions which have been demonstrated to be electrophoretically homogeneous in the sense that other components are

TABLE I

Composition of Immune Proteins

The data are given as gm. of constituent per 100 gm. of anhydrous protein. The values for bovine γ-2-globulin are averages for two preparations. Determinations for the bovine immune lactoglobulins are averages for two preparations of each, one from milk and one from colostrum.

Constituent	Human γ-globulin II-1 (60)	Human γ-globulin II-1,2 (61,62)	Human γ-globulin II-3 (61,62)	Bovine γ-2-globulin (61,62)	Bovine T-(γ-1)-globulin (61,62)	Bovine immune eulactoglobulin (61,62)	Bovine immune pseudolactoglobulin (61,62)	Equine γ-2-globulin (61,62)	Equine T(γ-1)-globulin (61,62)
Arginine	4.8	5.1	3.7	5.8	4.8	4.9	3.5; 5.6*; 5.2†	3.8	2.8
Aspartic Acid	8.8						9.4*; 9.3†		
Cystine (+cysteine)	3.1	2.6	2.7	2.9	2.8	3.2	3.0	2.6	2.5
Glutamic acid	11.8						12.3*; 10.7†		
Glycine	4.2								
Histidine	2.50	2.01	1.91	2.05	2.01	1.89	2.1; 2.3*; 2.2†	2.44	2.43
Isoleucine	2.7	2.8	2.0	3.2	3.0	3.1	3.1	4.4	3.3
Leucine	9.3	9.3	9.5	7.4	8.6	10.4	9.1; 8.5*; 8.3†	9.0	7.5
Lysine	8.1	7.2	6.3	6.7	6.4	6.3	7.2; 6.1*; 6.1†	8.6	6.7
Methionine	1.06	1.12	0.87	1.18	1.00	0.98	1.08; 1.3*; 1.1†	0.95	0.72
Phenylalanine	4.6	4.5	4.7	3.2	4.5	3.6	3.8; 3.9*; 4.3†	4.4	4.1
Proline	8.1						10.0*		
Serine	11.4								
Threonine	8.4	8.8	7.4	10.0	9.5	10.5	10.1; 9.0*; 10.2†	11.1	8.7
Tryptophan	2.86	2.6	2.8	2.6	2.6	2.4	2.7; 3.2*	2.7	2.8
Tyrosine	6.75						6.7*	6.8‡	6.8‡
Valine	9.7	9.7	9.7	10.0	9.5	10.4	9.4; 8.7*; 9.3†	10.1	10.4
Hexose		2.3	2.3	2.1	2.5	2.9	2.8	2.5	2.6
Hexosamine		1.27	1.23	1.31	1.50	1.45	1.32	1.18	1.53

* Data of Hansen, Potter & Phillips (64).
† Data of Henderson & Snell (65).
‡ Unpublished observations of Jager & Smith.

not detectable. However, since there is greater spreading of the migrating boundaries than expected for monodisperse systems, each fraction is undoubtedly a mixture of very similar molecules. Although data for alanine have not yet been reported, sufficient information is available to give a general picture of these proteins. It must be stated that no really unique feature in composition is apparent from these data. The data given are for immune fractions from both normal and hyperimmune animals; no recent analyses have been published for antibodies obtained by specific precipitation. It would be interesting to have such data for antibodies and γ-globulins of the rabbit, the most commonly used species for immunochemical studies.

The studies of Porter (66) have shown that normal rabbit γ-globulin and the specifically precipitable antibody to egg albumin contain a single free α-amino group, that of alanine, and possess the identical terminal peptide sequence, alanylleucylvalylaspartylglutamyl. The two preparations have identical amounts of reactive ϵ-amino groups of lysine. The percentage of unreactive histidine to total histidine was greater in the γ-globulin-egg albumin precipitate than in the γ-globulin. This could imply that an imidazole group of the antibody participated in the specific antigen-antibody complex or, alternatively, that three imidazole groups in the ovalbumin were blocked in the specific precipitate.

Immunochemical Properties of Antibodies

Thus far, attempts to find the differences between antibodies and normal γ-globulins have not been very revealing. That small physical and chemical differences, e.g., electrophoretic mobility, are found among different antibodies does not necessarily indicate major alterations between specific antibodies and globulins of the same electrophoretic mobility. Perhaps great differences are not to be expected if antibodies differ only slightly in specific surface configuration at the active sites. Specific binding of molecules with proteins in general has been shown to depend on particular sites, especially in the case of enzymes which possess well-defined prosthetic groups. The specificity of antigen-antibody reactions is certainly as high as those of enzyme reactions, as shown by the classical studies of Landsteiner and of many others by chemical modification of antigens and inhibition of antigen-antibody reactions with low molecular weight haptens.

An interesting example of the analogy of enzymic reactions and antibody reactions is provided by the work of Bowman, Mayer & Rapp (67). They present quantitative data which indicate that in the presence of an excess amount of complement, the antibody hemolysin is able to combine with a red blood cell to induce lysis, then to dissociate from this complex and to become available for combination with and lysis of another erythrocyte. This is a catalytic reaction comparable to that observed in the reaction of enzyme and substrate.

Some information concerning the nature of the reactive group on the globulin molecule may be gleaned by chemical modification of antibody or

* Data of Hansen, Potter & Phillips (64).
† Data of Henderson & Snell (65).
‡ Unpublished observations of Jager & Smith.

antigen and examination of the effect of the induced changes on the interaction of antigen and antibody. Only a few examples of such studies can be mentioned.

By the action of proteolytic enzymes, immune globulins may be cleaved in half or into smaller fragments without impairing the antibody content of the fraction. Equine diphtheria antitoxin has been obtained in highly purified form after treatment with pepsin (63), and after treatment with trypsin, Northrop (68) succeeded in isolating a crystalline antitoxin, the first preparation obtained in this form. These digested antitoxins are approximately half the normal size, having molecular weights of about 90,000 (63, 69). Other immune globulin fractions may also be digested by such proteolytic enzymes as pepsin, trypsin, papain, bromelin etc. without losing the specific antibody activity. This work, which has recently been reviewed in detail by Williams (4), demonstrates that the entire antibody molecule is not essential for immunological activity; however, the experiments of Hartley (47), cited above, indicate an important biological difference between digested and undigested antitoxins.

Although the complex of diphtheria toxin and antitoxin may be digested by proteolytic enzymes leaving the half-molecules of antitoxin, it is noteworthy that Northrop & Goebel (33) found the complex of antibody with pneumococcal polysaccharide to be resistant to the action of trypsin.

Porter (66) treated rabbit antibody to egg albumin with thioglycollate, which acts as a reducing substance. The modified antibody still reacted with antigen but the flocculation time was increased. Such quantitative alterations have frequently been reported. In an earlier study, Smetena & Shemin (70) irradiated antiserum to egg albumin with ultraviolet and visible light in the presence of hematoporphyrin and observed some decrease in the amount of precipitate formed when this treated antiserum was added to antigen. Wagley *et al.* (71) treated an anti-Rh serum with plant peroxidase and hydrogen peroxide with resultant reduction in agglutinating activity. The treated antiserum had an altered ultraviolet absorption spectrum suggesting a modification in aromatic amino acids. The treated serum after hydrolysis was found on microbiologic assay to contain less tyrosine than untreated serum.

Antisera may be tagged with small amounts of I^{131} and still retain their ability to react with antigen in the manner of uniodinated antisera [Masouredis *et al.* (72); Cohen (73); Banks *et al.* (74)]. Likewise, Eisen & Keston (75) found that bovine serum albumin labeled with I^{131} reacts with antisera to native albumin in a similar quantitative manner. Such observations have permitted tracer studies of the fate, distribution, and half-life of labeled antigens and antibodies since the radioactivity provides convenient methods of measurement.

Antigens also may undergo varying degrees of alteration with differing effects on their reactivity with antiserum to untreated antigen. Mayer & Heidelberger (76) compared the reactivity of normal and phosphorylated

horse serum albumin with antisera to these two antigens and found quantitative differences. The antisera obtained from this phosphorylated antigen did not react with phosphorylated egg albumin. They indicate that substitution on a protein molecule by a reactive group may produce local changes on the surface or general alterations in the structure of the protein molecule as a result of rupture of certain bonds; either change could alter the immunological specificity of a treated antigen.

Heidelberger, MacLeod & DiLapi (77) demonstrated that alkaline degradation of certain pneumococcal polysaccharides impaired their ability to react *in vitro* with antiserum but did not alter their ability to stimulate antibody formation to intact polysaccharide in man. Erickson & Neurath (78) reported earlier that regenerated horse serum albumin after treatment with 8 *M*. urea gave essentially the same amount of precipitate as did addition of an equivalent amount of untreated antigen when added to rabbit antiserum against native serum albumin. Fleming, Moore & Butler (79) found that protein treated with *bis*-β-chloroethyl sulfide in the presence of phosphate acquired marked specificity to the group added to the protein, whereas the same compound in the absence of phosphate did not confer group specificity.

Immunological homogeneity.—When a "homogeneous" antigen is added to its homologous antiserum, the amount of precipitate formed increases progressively with increasing quantities of antigen up to the point at which the amount of antigen added is sufficient to remove all of the specific antibody from the antiserum; this is the zone of equivalence. Heidelberger & Kendall (80, 81, 82) demonstrated that the relation between the amount of antigen added (An) and the amount of antibody precipitated (Ab) may be expressed by the equations:

$$(\mathrm{Ab}/\mathrm{An}) = 2\mathrm{R} - \frac{\mathrm{R}^2\mathrm{An}}{\mathrm{A}}$$

or

$$(\mathrm{Ab}/\mathrm{An}) = 3\mathrm{R} - 2[\mathrm{R}^3\mathrm{An}/\mathrm{A}]^{1/2}$$

where R is the ratio of antibody/antigen at the zone of equivalence and A is the total amount of antibody in the system. When an animal is subjected to repeated courses of immunization, it is found that the value for R may increase. This is frequently ascribed to the fact that the antigen molecule has more than one antigenic group on its surface so that with prolonged immunization, antibody is formed to additional groups on the antigen surface.

The quantity of precipitate formed when a given amount of antigen is added to a specific amount of rabbit antiserum is relatively uninfluenced by moderate variations in temperature and pH, but increasing concentrations of salt produce a decrease in the amount of precipitate. In contrast, Goodman, Wolfe & Norton (83) report that when chicken antiserum reacts with antigen, the amount of precipitate increases progressively with increasing concentrations of sodium chloride up to 8 per cent (w./v.). Krueger & Heidel-

berger (84) removed lipids by ether and alcohol extraction at low temperatures from horse antisera to rabbit albumin, rabbit globulin, and to type III pneumococcus. With the quantitative precipitin technique, it was shown that such antisera react completely with added antigen, the time required being somewhat longer and the rate of centrifugation having to be somewhat more rapid than for unextracted antiserum. The initial combining ratios (2R or 3R in the previous equations) appear to be altered in the extracted antisera.

Cross-reactions in which an antiserum to one antigen reacts with a heterologous antigen illustrate some of the difficulties in the evaluation of quantitative precipitin studies. These cross-reactions imply reactions with single different antigens, not with mixed antigenic substances. Examples of cross-reactions are those encountered between type III pneumococcus antiserum and type VIII pneumococcal polysaccharide found by Heidelberger, Kabat & Mayer (85), cross-reactions among the mammalian thyroglobulins shown by Stokinger & Heidelberger (86), and between vegetable gums with type II antipneumococal serum observed by Marrack & Carpenter (87). With the quantitative precipitin method, Kabat *et al.* (88) showed species differences among the various blood group substances. Shedlovsky & Elliott (89) describe an interesting situation in which an antiserum to a streptococcal proenzyme reacts both with proenzyme and active proteinase whereas antiserum to active proteinase combines only with this specific antigen.

It has frequently been observed that individual antisera differ in their degree of cross-reactivity with other distinct antigens. In some instances, cross-reactivity increases with prolonged immunization (86). As Marrack & Carpenter (87) have indicated, such cross-reactions could be ascribed to the fact that the antiserum contains qualitatively different types of antibody molecules which react with different antigenic groups. Alternatively, the antibody molecules could differ in degree or rate of reactivity but not in kind; this latter hypothesis is favored by these investigators. However, injection of an antigen containing two or three different kinds of determinant groups leads to the formation of different types of antibodies as shown by Haurowitz & Schwerin (90); also see Cann *et al.* (32). Haurowitz favors the view that an immune serum will contain a variety of antibodies even when a single type of antigen is used because of the presence of several determinant groups in an individual molecule.

Until very recently, it was believed to be relatively simple to test for the immunological homogeneity of an antigen and its antiserum. The procedure consisted of adding increasing amounts of antigen to a constant amount of antiserum, measuring the quantity of precipitate formed in each tube and examining the supernatant by addition of antigen to one aliquot, antiserum to another. With a homogeneous system, the supernatant fluid would react only with antigen or with antiserum and not with both. The ratio for antibody precipitated to antigen added in the zone of antibody excess would give a straight line when plotted against antigen or $(\text{antigen})^{1/2}$. On this basis, various proteins and carbohydrates appeared to react as homogeneous sub-

stances with their individual antisera. The validity of these tests as the only criteria of immunological homogeneity has been made questionable by the work of Cohn, Wetter, & Deutsch (91), who studied the quantitative precipitin reaction of egg albumin and conalbumin to specific antisera. When increasing quantities of antigen were added to a constant quantity of antiserum, the usual increase in precipitate up to the zone of equivalence was found. On addition of larger quantities of antigen, in the zone of antigen excess, the amount of precipitate remained high over a broad zone. However, after selective absorption of the antiserum, there was a prompt decrease in the amount of precipitate in the zone of antigen excess. They ascribe the persistence of precipitate beyond the zone of equivalence to contaminating antibodies and contaminating antigens.

Cohn & Pappenheimer (35) studied quantitatively the precipitate formation between diphtheria toxin and antitoxin formed in man, monkey, guinea pig, and rabbit. After absorption with P-proteins, the precipitin curves all fell sharply in the zone of antigen excess. In a few instances, unabsorbed antisera were examined. These were found to have higher values for R (ratio of antibody to antigen at equivalence) and to show considerable amounts of precipitate far in the zone of antigen excess. In a subsequent study, Cohn, Deutsch & Wetter (92) examined the reaction of certain antisera to human γ-globulin fractions with their specific antisera. They concluded on the basis of the sustained precipitate in the zone of antigen excess, that these fractions were immunologically heterogeneous.

Another useful procedure for determining heterogeneity (mixed antigens and antibodies) in an immunologic system is the method of agar diffusion developed by Oudin (93, 94). Also see Ouchterlony (95, 96, 97), Elek (98), and Munoz & Becker (99). In the procedure of Oudin, antiserum is added to a solution of agar and allowed to gel in a small tube. The agar gel is then overlaid with an aqueous solution of antigen. Antigen diffusing into the agar-containing antibody gives a visible precipitate, the boundary extending with time. In accord with diffusion theory, when the linear movement of antigen is plotted against $(\text{time})^{1/2}$, a straight line results. The slope of this line is influenced by temperature, the concentration of antigen and of the specific antibody, and upon the diffusion constant of the antigen. If only the concentration of antigen is varied in a system of a single antigen and a single antibody, the slope of the line is proportional to the logarithm of the antigen concentration. When both antigen and antibody concentration are known, Becker *et al.* (100) have reported that it is possible to calculate the diffusion constant for the antigenic protein used. Previously, Ouchterlony (96, 97) had shown that the ratio of the diffusion constants of antigen and antibody could be calculated for a similar system and the constants are given for diphtheria toxin and antitoxin. Independent studies by Smith & Jager (101) have shown that the equations developed by Adair (102) for the diffusion of low molecular weight electrolytes are applicable to the antigen-antibody system. The diffusion constants for egg albumin, bovine serum albumin,

and human γ-globulin are in reasonable agreement (±10 per cent) with those obtained by other methods.

Of considerable importance for the study of homogeneity is the observation that when the antigen is inhomogeneous and the antiserum in agar contains several antibodies, two or more boundary zones will be observed if the antigens have different diffusion rates. In spite of its great value, certain obvious limitations exist in this system. Thus, if a lighter (more rapidly diffusing) and a heavier antigen were allowed to diffuse through agar, and if the two antigens cross-reacted with the same antibody, there would be only one boundary zone since the more rapidly moving component could remove all the antibody. Another difficulty is the impossibility of detecting impurities present in very low concentration. Thus, if an antiserum which contained two antibodies in equal proportions were overlaid with a mixed antigen, containing one component as 99 per cent, the other as 1 per cent of the protein, the more concentrated antigen, even though it be the more slowly diffusing component, may by virtue of its concentration obscure a second antigen of more rapid diffusion rate because of its relatively low concentration. As an example, evidence can be cited from a study (103) of rabbit antisera to human γ-globulin (Fraction II-1,2). This system gives only one visible band when Fraction II-1,2 or when whole human serum is added as antigen. When, however, a solution containing primarily α- and β-globulins is used as the test antigen, two distinct bands are seen in the antibody-containing agar layer (103). Here it is assumed that the mixture of α- and β-globulins contains the contaminating or cross-reacting antigens in sufficient quantities to give visible bands, by virtue of a more rapid diffusion rate possessed by the higher concentrations of antigens.

Specific antibodies, being globulins, are themselves antigenic. Ideally this situation would be examined by injecting pure antibody globulin of one species into another and then studying the quantitative characteristics of this antiserum with the original antibody globulin used as antigen. In practice it has been found more convenient to immunize an animal with the specific precipitate of antigen and antibody. The antiserum obtained is then tested against specific precipitates made with antiserum of the same species combined with a different antigen. It appears that the specific component or components of the antibody molecule that determine its specificity do not modify the characteristics of an antibody made to a specifically reacting antibody. Treffers & Heidelberger (104) immunized rabbits with the specific precipitate of type I antipneumococcal horse serum combined with the specific polysaccharide. Specific precipitates of horse antiserum to pneumococcus type I, type II, or type VIII, or *Hemophilus influenzae*, all removed essentially the same amount of antibody from the rabbit antiserum. Subsequently, these investigators (105) showed that chicken antisera to the horse serum precipitates did not distinguish between type I and type II pneumococcal precipitates. The same phenomena were noted with rabbit specific precipitates in the chicken. However, rabbit antisera to specific precipitates

of horse antisera with pneumococcal polysaccharides showed quantitatively different responses with horse toxin-antitoxin precipitates. In general, the toxin-antitoxin precipitates removed only about 60 per cent of the total antibody present in rabbit serum to a horse antiserum-pneumococcal polysaccharide precipitate. This indicates immunologic differences between the so-called flocculating and the precipitin or polysaccharide type of antibody in the horse (104).

This leads to a consideration of the immunologic properties of immune globulin fractions, not specific antibodies. Treffers, Moore & Heidelberger (106) injected rabbits with specific precipitates of equine antiserum with type II pneumococcal polysaccharide. The rabbit antiserum gave maximal precipitation when treated with the specific precipitate. However, moderate amounts of antibody were removed from the rabbit antiserum by addition of normal γ-globulin and normal β-globulin separated by electrophoresis from horse serum. Jager and co-workers (107) studied the immunologic cross-reactions of horse γ- and T-globulins as well as of a heterogeneous fraction, consisting primarily of β-globulin. All three fractions showed considerable cross-reactions as judged by quantitative precipitin studies.

Perhaps the greatest interest has centered in the study of antibodies to human γ-globulin fractions. If an antiserum to human γ-globulin could be found to be specific for this component, then the precipitin technique could be employed to determine quantitatively the amount of γ-globulin in extremely small amounts of normal and abnormal human serum and other body fluids. Many investigators have shown that a similar method offers a reasonably accurate means for determining the albumin content of human body fluids.

As mentioned earlier, human γ-globulin may be separated chemically into two fractions of different mobilities, termed γ_1 and γ_2. Each fraction contains more than one component when examined in the ultracentrifuge. The use of electrophoresis-convection permits further separation of these fractions. Chemically, with Method 9 of Oncley *et al.* (14), the γ_2-globulin is obtained in Fractions II-1,2 and II-3 which differ in their isoelectric points and in the relative amounts of water-soluble and water-insoluble components.

As regards γ_2-globulin, Kendall (108) prepared a pseudoglobulin fraction of human serum which behaved as a homogeneous antigen as judged by the criterion of a single component in the supernatant after varying quantities of antigen had been added to fixed amounts of antibody. Jager *et al.* (109) immunized rabbits to Fractions II-1,2 and II-3 and found the two antigens to behave quantitatively the same when added either to II-1,2 or II-3 rabbit antiserum. No studies were made in the zone of antigen excess. Kabat & Murray (110), in a similar study, found that Fractions II-1,2, II-3, a human pseudoglobulin fraction obtained by precipitation from serum with 33 per cent saturated ammonium sulfate, and an additional sample of human γ-globulin supplied by Brand, all behaved quantitatively alike in the

amount of precipitate they removed from antisera to γ-globulin. They also studied the γ_1 fraction of Nichol & Deutsch (16) and found it to contain about 60 per cent by weight of a substance immunologically equivalent to γ_2-globulin. All the antibody could be removed from γ_2 antiserum by adding a sufficient amount of the γ_1-globulin. Kabat & Murray also indicated that their antisera to γ_2-globulin were homogeneous by examination of the supernatant as well as by agar diffusion studies.

Cohn, Deutsch & Wetter (92) made an extensive study of rabbit and chicken antisera to γ_1- and γ_2-globulins as well as Fractions II-1,2 and II-3. None of their antisera was homogeneous as judged by their criterion of a narrow zone of equivalence with rapid decline of amount of precipitate in the zone of antibody excess. In this zone, they were able to show for rabbit antisera, a difference between the II-1,2 and II-3 globulins. Moreover, they observed distinct immunologic differences between γ_1- and γ_2-globulins.

More recently, this problem has been pursued by Jager & Smith (103) in a study of six individual rabbit antisera to Fraction II-1,2 and three antisera to Fraction II-3. In each instance, the analysis of supernates has demonstrated only antigen or antibody. All have given a linear relationship in the zone of antibody excess when the ratio of antibody to antigen is plotted against increasing quantities of antigen. Each antiserum in an agar gel was covered with a solution of Fractions II-1,2; II-3; or whole human serum and, in each instance, one band of precipitate was observed. When, however, the test antigen was a mixture of α- and β-globulins, two distinct bands were frequently found with the agar diffusion method. In all instances, studies were made in the zone of antigen excess using both II-1,2 and II-3 antigens. In agreement with Cohn *et al.* (92), it was found that the amount of precipitate in the zone of antigen excess remains near maximal values for a wide zone. Adsorption of the antisera with a mixture of α- and β-globulins results in a decrease in the value for R (antibody/antigen at equivalence). Comparative studies in the zone of antigen excess with II-1,2 or II-3 rabbit antisera only occasionally have shown significant differences between the amounts of precipitate to these two antigens whether the antiserum be made to II-1,2 or II-3 antigens; this is not in accord with the observations of Cohn *et al.* (92).

As regards the use of calibrated antiserum to γ-globulin for the estimation of γ-globulin in whole human serum, there is some disagreement. The values for γ-globulin by this procedure were considerably higher than expected from electrophoretic observations in the study of Kendall (108). Similarly, Jager *et al.* (109) and Kunkel and co-workers (111) found the values for γ-globulin by the quantitative precipitin technique to be greatly in excess of the values determined electrophoretically. However, Bendich & Kabat (112) found good agreement between immunologic and electrophoretic estimates of γ-globulin. Kibrick & Blonstein (113), using antisera to γ-globulin that had been absorbed with α- and β-globulins, found good agreement between the immunologic procedure and a chemical method which

they had compared with electrophoretic measurements. Marrack, Johns & Hoch (114) report good agreement between electrophoretically and immunologically determined γ-globulin for two out of three human sera using rabbit antisera absorbed with electrophoretically-isolated α- and β-globulins. From recent observations (103), it would appear that with suitably absorbed antisera, better agreement can be obtained between γ-globulin determined immunologically and those obtained electrophoretically than with unabsorbed antisera.

Myeloma proteins.—It has frequently been demonstrated that in patients with multiple myeloma, a malignant tumor of plasma cells, there is a striking increase in the serum γ-globulin, β-globulin, or in "M-protein," a globulin intermediate in mobility between β- and γ-globulin. The mobility of the so-called "M-protein" is probably identical with that of γ_1-globulin. Earlier studies of these myeloma proteins have been summarized in an excellent review by Gutman (115).

Studies of these proteins have aroused considerable interest because of their value in clinical diagnosis and because an important role in antibody production has been ascribed to the plasma cells by some investigators. The complex problem of the sites of antibody production and release is beyond the scope of this article; discussion from various viewpoints may be found in the reviews of Ehrich (116), Fagreus (117), and White (118).

The abnormal proteins present in the sera of myeloma cases appear to fall into two classes according to size, regardless of electrophoretic mobility: those proteins with sedimentation constants of about 7 S (molecular weight of 160,000 to 180,000), which closely resemble ordinary γ-globulins, and proteins with 3 to 4 S having molecular weights of 30 to 40,000 (119). These latter proteins are closely related to, if not identical with, the Bence-Jones proteins found in the urine of myeloma cases. Indeed, it has been demonstrated by immunochemical techniques (119) that the small abnormal serum protein gives a strong precipitin reaction with rabbit antisera to urinary Bence-Jones protein.

Rundles *et al.* (120) have reported a detailed study of the abnormal serum and urinary constituents of 30 patients with multiple myeloma. The proteins were characterized by physical methods. As in earlier studies, the abnormal serum proteins usually had a molecular weight of about 150,000 whereas that of the urinary proteins was between 30 and 40,000. These authors suggest that proliferating plasma cells produce a protein of abnormal homogeneity, and then by cleavage *in vivo*, fractions are formed which are filtered through the glomeruli and constitute the urinary Bence-Jones proteins. This is in contrast to earlier theories (see 115) ascribing the increase in γ-globulins to the formation of antibodies to the Bence-Jones proteins. Schoenbach and co-workers (121) have reported an electrophoretic analysis of an extract of a localized myeloma (plasma cell tumor) which had 57 per cent of a component with the mobility of γ-globulin.

Kunkel, Slater & Good (111) have found that antisera against prepara-

tions of normal human γ-globulin gave precipitates with the myeloma proteins obtained from the sera of four patients with multiple myeloma. Absorption of γ-globulin antiserum with purified myeloma proteins removed only a portion of the total antibody. Antiserum prepared against a highly purified myeloma protein, furnished by Petermann and Cogin, gave a precipitin reaction with Fraction II-1,2 although it was less reactive than the myeloma protein; this myeloma protein was 98 per cent homogeneous electrophoretically and showed 95 per cent of a component with s = 7 S in the ultracentrifuge. Although quantitative differences were found between the normal γ-globulins and these myeloma proteins, there appears to be no doubt of the γ-globulin character of certain of these abnormal proteins. However, these investigators studied one myeloma protein of high electrophoretic mobility whose antisera failed to react with either normal γ-globulin antisera or antisera to other myeloma proteins. A similar finding has also been reported by Wuhrmann *et al.* (122), who prepared an antiserum to a myeloma protein of high mobility; the antiserum failed to react with normal serum components.

LITERATURE CITED

1. Kabat, E. A., *J. Immunol.*, **47**, 513–87 (1943)
2. Treffers, H. P., *Advances in Protein Chem.*, **1**, 69–119 (1944)
3. Campbell, D. H., *Ann. Rev. Microbiol.*, **2**, 269–78 (1948)
4. Williams, J. W., *Fortschr. chem. organ. Naturstoffe*, **7**, 270–305 (1950)
5. Mayer, M. M., *Ann. Rev. Biochem.*, **20**, 415–40 (1951)
6. Longsworth, L. G., *Chem. Revs.*, **30**, 323–39 (1942)
7. Tiselius, A., and Kabat, E. A., *J. Exptl. Med.*, **69**, 119–31 (1939)
8. Kekwick, R. A., and Record, B. R., *Brit. J. Exptl. Path.*, **22**, 29–44 (1941)
9. van der Scheer, J., Wyckoff, R. W. G., and Clarke, F. G., *J. Immunol.*, **39**, 65–71 (1940)
10. Moore, D. H., van der Scheer, J., and Wyckoff, R. W. G., *J. Immunol.*, **38**, 221–30 (1940)
11. Deutsch, H. F., Nichol, J. C., and Cohn, M., *J. Immunol.*, **63**, 195–210 (1949)
12. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, **68**, 459–75 (1946)
13. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittleman, D., Mouton, R. F., Schmid, K., and Uroma, E., *J. Am. Chem. Soc.*, **72**, 465–74 (1950)
14. Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W., and Gross, P. M., Jr., *J. Am. Chem. Soc.*, **71**, 541–50 (1949)
15. Deutsch, H. F., Gosting, L. J., Alberty, R. A., and Williams, J. W., *J. Biol. Chem.*, **164**, 109–18 (1946)
16. Nichol, J. C., and Deutsch, H. F., *J. Am. Chem. Soc.*, **70**, 80–83 (1948)
17. Kirkwood, J. G., *J. Chem. Physics*, **9**, 878–79 (1941)
18. Cann, J. R., Brown, R. A., and Kirkwood, J. G., *J. Biol. Chem.*, **181**, 161–70 (1949)
19. Cann, J. R., Kirkwood, J. G., Brown, R. A., and Plescia, O. J., *J. Am. Chem. Soc.*, **71**, 1603–8 (1949)
20. Cann, J. R., Brown, R. A., and Kirkwood, J. G., *J. Am. Chem. Soc.*, **71**, 2687–91 (1949)
21. Cann, J. R., Brown, R. A., and Kirkwood, J. G., *J. Am. Chem. Soc.*, **71**, 1609–14 (1949)
22. Cann, J. R., Brown, R. A., Kirkwood, J. G., and Hink, J. H., Jr., *J. Biol. Chem.*, **185**, 663–67 (1950)
23. Cann, J. R., and Kirkwood, J. G., *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 9–23 (1950)
24. Treffers, H. P., Heidelberger, M., and Freund, J., *J. Exptl. Med.*, **86**, 83–94 (1947)
25. Heidelberger, M., *J. Exptl. Med.*, **86**, 77–81 (1947)
26. Treffers, H. P., Heidelberger, M., and Freund, J., *J. Exptl. Med.*, **86**, 95–106 (1947)
27. Sandor, G., and Skrobisz, C., *Ann. inst. Pasteur*, **73**, 1041–42 (1947)
28. Sandor, G., and Ceddaha, L., *Bull. soc. chim. biol.*, **31**, 1005–18 (1949)
29. Smith, E. L., and Gerlough, T. D., *J. Biol. Chem.*, **167**, 679–87 (1947)
30. Deutsch, H. F., and Nichol, J. C., *J. Biol. Chem.*, **176**, 797–812 (1948)
31. Hess, E. L., and Deutsch, H. F., *J. Am. Chem. Soc.*, **71**, 1376–81 (1949)
32. Cann, J. R., Campbell, D. H., Brown, R. A., and Kirkwood, J. G., *J. Am. Chem. Soc.*, **73**, 4611–15 (1951)

33. Northrop, J. H., and Goebel, W. F., *J. Gen. Physiol.*, **32,** 705–24 (1949)
34. Enders, J. F., *J. Clin. Invest.*, **23,** 510–30 (1944)
35. Cohn, M., and Pappenheimer, A. M., Jr., *J. Immunol.*, **63,** 291–312 (1949)
36. Hink, J. H., Jr., and Johnson, F. F., *J. Immunol.*, **64,** 39–43 (1950)
37. Tauber, H., McLeod, C., Garson, W., and Magnuson, H. J., *Proc. Soc. Exptl. Biol. Med.*, **78,** 110-12 (1951)
38. Davis, B. D., Moore, D. H., Kabat, E. A., and Harris, A., *J. Immunol.*, **50, 1–20** (1945)
39. Cooper, J. A., *Proc. Soc. Exptl. Biol. Med.*, **57,** 248–52 (1944); *J. Investigative Dermatol.*, **6,** 109-12 (1945)
40. Cann, J. R., Brown, R. A., Gajdusek, D. C., Kirkwood, J. G., and Sturgeon, P., *J. Immunol.*, **66,** 137–44 (1951)
41. Campbell, D. H., Cann, J. R., Friedman, T. B., and Brown, R. A., *J. Allergy*, **21,** 519–21 (1950)
42. Florman, A. L., Schick, B., and Scalettar, H. E., *Proc. Soc. Exptl. Biol. Med.*, **78,** 126–28 (1951)
43. Adamson, C. A., Löfgren, S., and Malmnäs, C., *Scand. J. Clin. Lab. Invest.*, **3,** 52–57 (1951)
44. Longsworth, L. G., Curtis, R. M., and Pembroke, R. H., Jr., *J. Clin. Invest.*, **24,** 46–53 (1945)
45. Brambell, F. W. R., Hemmings, W. A., Henderson, M., Parry, H. J., and Rowlands, W. T., *Proc. Roy. Soc.* (*London*), [B]**136,** 131–44 (1949)
46. Brambell, F. W. R., Hemmings, W. A., Henderson, M., and Rowlands, W. T., *Proc. Roy. Soc.* (*London*), [B]**137,** 239–52 (1950)
47. Hartley, P., *Proc. Roy. Soc.* (*London*), [B]**138,** 499–513 (1951)
48. Sternberger, L. A., and Pressman, D., *J. Immunol.*, **65,** 65–73 (1950)
49. Sternberger, L. A., and Petermann, M. L., *J. Immunol.*, **67,** 207–12 (1951)
50. Kabat, E. A., *J. Exptl. Med.*, **69,** 103–18 (1939)
51. Campbell, D. H., Luescher, E., and Lerman, L. S., *Proc. Natl. Acad. Sci. U. S.*, **37,** 575-78 (1951)
52. Svensson, H., *Arkiv Kemi, Mineral. Geol.*, [A]**17,** 1–8 (1943)
53. Williams, J. W., Petermann, M. L., Colovos, G. C., Goodloe, M. B., Oncley, J. L., and Armstrong, S. H., Jr., *J. Clin. Invest.*, **23,** 433–36 (1944)
54. Oncley, J. L., Scatchard, G., and Brown, A., *J. Phys. & Colloid Chem.*, **51,** 184–98 (1947)
55. Smith, E. L., *J. Biol. Chem.*, **165,** 665–76 (1946)
56. Smith, E. L., and Brown, D. M., *J. Biol. Chem.*, **183,** 241–49 (1950)
57. Savat, R. S. (Batchelor's thesis, Univ. of Wisconsin, 1950)
58. Cann, J. R., Brown, R. A., Singer, S. J., Shumaker, J. B., Jr., and Kirkwood, J. G., *Science*, **114,** 30–33 (1951)
59. Pedersen, K. O., *Ultracentrifugal Studies on Serum and Serum Fractions* (Almqvist and Wiksells Boktryckeri A.-B., Upsala, Sweden, 178 pp., 1945)
60. Brand, E., Kassel, B., and Saidel, L. J., *J. Clin. Invest.*, **23,** 437–44 (1944); Brand, E., *Ann. N. Y. Acad. Sci.*, **47,** 187–228 (1946)
61. Smith, E. L., Greene, R. D., and Bartner, E., *J. Biol. Chem.*, **164,** 359–66 (1946)
62. Smith, E. L., and Greene, R. D., *J. Biol. Chem.*, **171,** 355–62 (1947)
63. Pappenheimer, A. M., Jr., Lundgren, H. P., and Williams, J. W., *J. Exptl. Med.*, **71,** 247–62 (1940)
64. Hansen, R. G., Potter, R. L., and Phillips, P. H., *J. Biol. Chem.*, **171,** 229–32 **(1947)**

65. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172,** 15–29 (1948)
66. Porter, R. R., *Biochem. J.*, **46,** 473–78 (1950)
67. Bowman, W. M., Mayer, M. M., and Rapp, H. J., *J. Exptl. Med.*, **94,** 87–110 (1951)
68. Northrop, J. H., *J. Gen. Physiol.*, **25,** 465–85 (1942)
69. Rothen, A., *J. Gen. Physiol.*, **25,** 487–96 (1942)
70. Smetena, H. and Shemin, D., *J. Exptl. Med.*, **73,** 223–42 (1941)
71. Wagley, P. F., Sizer, I. W., Diamond, L. K., and Allen, J. H., *J. Immunol.*, **64,** 85–94 (1950)
72. Masouredis, S. P., Melcher, L. H., and Koblick, D. C., *J. Immunol.*, **66,** 297–302 (1951)
73. Cohen, S., *J. Immunol.*, **67,** 339–46 (1951)
74. Banks, T. E., Francis, G. E., Mulligan, F. W., and Wormall, A., *Biochem. J.*, **48,** 180–87 (1951)
75. Eisen, H. N., and Keston, A. S., *J. Immunol.*, **63,** 71–80 (1949)
76. Mayer, M. M., and Heidelberger, M., *J. Am. Chem. Soc.*, **68,** 18–25 (1946)
77. Heidelberger, M., MacLeod, C. M., and DiLapi, M. M., *J. Immunol.*, **66,** 145–49 (1951)
78. Erickson, J. O., and Neurath, H., *J. Exptl. Med.*, **78,** 1–8 (1943)
79. Fleming, D. S., Moore, A. M., and Butler, G. C., *Biochem. J.*, **45,** 546–52 (1949)
80. Heidelberger, M., and Kendall, F. E., *J. Exptl. Med.*, **62,** 467–83 (1935)
81. Heidelberger, M., and Kendall, F. E., *J. Exptl. Med.*, **62,** 697–720 (1935)
82. Kendall, F. E., *Ann. N. Y. Acad. Sci.*, **43,** 85–105 (1942–1943)
83. Goodman, M., Wolfe, H. R., and Norton, S., *J. Immunol.*, **66,** 225–36 (1951)
84. Krueger, R. C., and Heidelberger, M., *J. Exptl. Med.*, **92,** 383–91 (1950)
85. Heidelberger, M., Kabat, E. A., and Mayer, M. M., *J. Exptl. Med.*, **75,** 35–47 (1942)
86. Stokinger, H. E., and Heidelberger, M., *J. Exptl. Med.*, **66,** 251–72 (1937)
87. Marrack, J., and Carpenter, B. R., *Brit. J. Exptl. Path.*, **19,** 53–65 (1938)
88. Kabat, E. A., Baer, H., Day, R. L., and Knaub, V., *J. Exptl. Med.*, **91,** 433–47 (1950)
89. Shedlovsky, T., and Elliott, S. D., *J. Exptl. Med.*, **94,** 363–72 (1951)
90. Haurowitz, F., and Schwerin, P., *Brit. J. Exptl. Path.*, **23,** 146–50 (1942); *J. Immunol.*, **47,** 111–19 (1943)
91. Cohn, M., Wetter, L. R., and Deutsch, H. F., *J. Immunol.*, **61,** 283–96 (1949)
92. Cohn, M., Deutsch, H. F., and Wetter, L. R., *J. Immunol.*, **64,** 381–95 (1950)
93. Oudin, J., *Ann. inst. Pasteur*, **75,** 33–51 (1948); *Ann. inst. Pasteur*, **75,** 109–29 (1948)
94. Oudin, J., *Comp. rend.*, **228,** 1890–92 (1949)
95. Ouchterlony, Ö., *Arkiv. Kemi, Mineral. Geol.*, [B]**26,** 1–9 (1948)
96. Ouchterlony, Ö., *Arkiv. Kemi*, **1,** 43–48 (1949)
97. Ouchterlony, Ö., *Arkiv Kemi*, **1,** 55–59 (1949)
98. Elek, S. D., *Brit. J. Exptl. Path.*, **30,** 484–500 (1949)
99. Munoz, J., and Becker, E. L., *J. Immunol.*, **65,** 47–58 (1950)
100. Becker, E. L., Munoz, J., Lapresle, C., and LeBeau, L., *Federation Proc.*, **10,** 401–2 (1951)
101. Smith, E. L., and Jager, B. V. (Unpublished data)
102. Adair, G. S., *Biochem. J.*, **14,** 762–79 (1920)
103. Jager, B. V., and Smith, E. L. (Unpublished data)
104. Treffers, H. P., and Heidelberger, M., *J. Exptl. Med.*, **73,** 125–40 (1941)

105. Treffers, H. P., and Heidelberger, M., *J. Exptl. Med.*, **73,** 293–97 (1941)
106. Treffers, H. P., Moore, D. H., and Heidelberger, M., *J. Exptl. Med.*, **75,** 135–50 (1942)
107. Jager, B. V., Smith, E. L., Bernhisel, H., and Jager, L. A., *J. Immunol.*, **65,** 311–22 (1950)
108. Kendall, F. E., *J. Clin. Invest.*, **16,** 921–31 (1937)
109. Jager, B. V., Smith, E. L., Nickerson, M., and Brown, D. M., *J. Biol. Chem.*, **176,** 1177–87 (1948)
110. Kabat, E. A., and Murray, J. P., *J. Biol. Chem.*, **182,** 251–59 (1950)
111. Kunkel, H. G., Slater, R. J., and Good, R. A., *Proc. Soc. Exptl. Biol. Med.*, **76,** 190–93 (1951)
112. Bendich, A., and Kabat, E. A., *J. Lab. Clin. Med.*, **34,** 1066–70 (1949)
113. Kibrick, A. C., and Blonstein, M., *J. Biol. Chem.*, **176,** 983–87 (1948)
114. Marrack, J. R., Johns, R. G. S., and Hoch, H., *Brit. J. Exptl. Path.*, **31,** 36–44 (1950)
115. Gutman, A. B., *Advances in Protein Chem.*, **4,** 155–250 (1948)
116. Ehrich, W. E., *Ann. N. Y. Acad. Sci.*, **46,** 823–57 (1946)
117. Fagreus, A., *Antibody Production in Relation to the Development of Plasma Cells* (Stockholm, Sweden, 122 pp., 1948)
118. White, A., *Ann. Rev. Physiol.*, **11,** 355–86 (1949)
119. Gutman, A. B., Moore, D. H., Gutman, E. B., McClellan, V., and Kabat, E. A., *J. Clin. Invest.*, **20,** 765–83 (1941)
120. Rundles, R. W., Cooper, G. R., and Willett, R. W., *J. Clin. Invest.*, **30,** 1125–42 (1951)
121. Schoenbach, E. B., Weissman, N., and Armistead, E. B., *J. Clin. Invest.*, **30,** 1125–42 (1951)
122. Wuhrmann, V. F., Wunderley, C., Hässig, A., and Hugentabler, F., *Helv. Med. Acta*, **16,** 279–82 (1949)

HAEMAGGLUTINATION IN RELATION TO HOST CELL-VIRUS INTERACTION[1]

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In this review the phenomena of haemagglutination by viruses will be considered only insofar as they bear on the process of interaction between the virus and the cell. Particularly in the case of the influenza viruses, haemagglutination has great practical importance for the recognition and titration of virus and for serological work. No more than incidental mention will be made of such matters. For various reasons, of which the most important is probably the unique suitability of infected embryo fluids for haemagglutination, work on the influenza viruses is more advanced than on any other group and this review will almost necessarily become essentially an account of the investigations that have been made on influenza and related viruses with only minor attention to other haemagglutinating viruses.

The recognition of haemagglutination by influenza virus was an early consequence of the development of methods of growing the virus in the amniotic (1) and allantoic (2, 3) cavities of the chick embryo. Both Hirst (4) and McClelland & Hare (5) independently observed that when blood and fluid from an infected embryo were accidentally mixed, the cells showed gross clumping. Hirst (6, 7, 8) rapidly sketched out the lines along which further study of the phenomena would develop both on the practical and on the theoretical side The first requirement for serious study was the development of a standard method for determining the haemagglutinin (HA) titre of an influenza virus preparation. Hirst (6) used a method depending on the extent to which red cells were removed from the supernatant fluid, but for most purposes workers have found it more convenient and sufficiently accurate to use the pattern produced by deposited cells in serial dilutions of virus (9, 10).

The main points established in Hirst's early work (6, 7) and abundantly confirmed since, were: (*a*) Haemagglutination is associated with adsorption of virus to the cells. (*b*) If allowance is made for the fact that the power to infect is more readily lost than the power to agglutinate red cells, HA titration gives a direct measure of the amount of virus present. (*c*) When agglutinated cells are held at 37° for some hours there is a gradual return of suspension stability with elution of the virus. The restabilized cells have lost the power to adsorb virus but the liberated virus retains its full haemagglutinating power.

These experiments and their subsequent elaboration by others made it clear that haemagglutination was a result of the adsorption of influenza

[1] The survey of the literature pertaining to this review was concluded in December, 1951.

virus particles to "receptor areas" on the red cells and that their subsequent elution was associated with enzymic destruction or inactivation of the receptor areas.

Subsequent research has been largely concerned with clarifying the function of the enzyme concerned, with identifying its substrate in the cell receptor, and with the application of findings from red cell studies to the problem of the interaction of virus with the surface of the susceptible cell.

Following the discovery of influenza virus haemagglutination, more or less similar reactions were shown to be produced by a considerable range of other viruses. In several instances the mechanism of haemagglutination is clearly very different from that of influenza virus.

The Range of Virus Haemagglutinins

All influenza viruses when present in adequate concentration agglutinate human and guinea pig erythrocytes (11), and all strains adapted to allantoic passage in the chick embryo produce some agglutination of chicken cells, in most cases to titres as high as those found with mammalian cells.

The viruses of mumps (12), fowl plague (13), and Newcastle disease of fowls (14) agglutinate human and chicken cells and make use of the same series of receptors as the influenza viruses. Mumps and Newcastle disease viruses show, however, some additional features in their ability to make irreversible union to the cell surface (15, 16, 17) and to cause haemolysis (18, 19, 20, 21).

The respiratory virus No. 1233 of Taylor (22), for which Francis (23) has suggested the name "Influenza C," is actively haemagglutinating for human and chicken cells but involves a different set of receptors (24)[2]. Pneumonia virus of mice (25, 26) agglutinates mouse and hamster cells under highly special conditions. Vaccinia (27, 28) and ectromelia (29) viruses produce a specific haemagglutinin which is not the virus particle and which agglutinates only cells from a proportion of chickens and pigeons.

Several viruses of the EMC group, including MM and SK-Columbia, agglutinate sheep cells (30, 31); there is some evidence that the receptor involved is the same as that concerned with influenza virus adsorption (32, 33). The Theiler group virus GD VII agglutinates human red cells in the cold and is reversibly eluted on warming (34).

Under very specific conditions Japanese encephalitis B virus agglutinates red cells from day-old chicks (35) and similar haemagglutinins are reported for St. Louis, West Nile, and Russian Spring-summer encephalitis viruses (36).

The Nature of Influenza Virus Haemagglutination

The essentials of the reaction between influenza virus and the erythrocyte are: (*a*) adsorption (*b*) elution and (*c*) subsequent modification of the

[2] Recent work by White (110) has shown that with human red cells influenza C virus takes its place in the normal receptor gradient between WS and LEE.

red cell surface. Hirst (7) interpreted this sequence as an enzymic action and all subsequent work has gone to substantiate the conception that an enzyme incorporated in the viral surface plays an essential part in the process.

The virus enzyme.—The character of an enzyme is defined by its substrate and the nature of the change induced in the substrate by its action. In the case of the influenza virus enzyme, advance toward a rigid characterization of this sort has been indirect and is still incomplete. There have been no reports of the separation of enzyme activity from the virus particles but soluble enzymes of essentially similar character have been obtained in culture filtrates from several bacterial species. It was shown by McCrea (37) that *Clostridium welchii* filtrates contained an enzyme capable of rendering red cells insusceptible to hemagglutination by influenza viruses. Soon afterwards it was found that much more active enzyme preparations could be obtained from *Vibrio cholerae* cultures (38, 39). The receptor destroying enzyme RDE is a typical soluble enzyme, highly concentrated and relatively pure preparations of which are now available (40). This enzyme has played an important part in subsequent work on the nature of haemagglutination and there is every reason to believe that in all essential respects it is identical with the enzyme of the influenza viruses.

A direct attempt to isolate and characterize the receptor or substrate on the red cell surface was made by de Burgh *et al.* in 1949 (41), but this approach has not been so fruitful as that which developed from the finding by Francis 1947 (42) that all normal sera contained a substance capable of inhibiting agglutination by heated influenza virus B.

If an allantoic fluid from an embryo infected two days previously with the strain LEE (influenza B) is heated to 55° for 30 min., it shows virtually no loss of haemagglutinin titre. The virus however is not released from the cells when they are maintained for some hours at 37°; i.e., the capacity of the virus to act as an enzyme has been lost (43). If such heated virus is used to titrate the antihaemagglutinating power of normal and immune sera using the serological techniques developed by Hirst (6) or Salk (10), very high values are obtained irrespective of the nature of the serum. The demonstration by Anderson (44) that this inhibitor in normal serum, often called Francis inhibitor, could be destroyed both by RDE and by active influenza virus showed that a substrate for their common enzyme could exist in soluble form. Soon afterwards it was found that similar inhibitory activity was present in a wide variety of physiological secretions and tissue extracts (45) containing mucoprotein or mucoid material. Some typical sources of high titre inhibitor are human cervical mucus (46) and meconium (47), egg-white (48, 49), and an extract from the submandibular salivary gland of the sheep (50, 51).

All these materials are complex mixtures and from none of them has a chemically and electrophoretically pure inhibitor been isolated. All the evidence (52, 53, 54) however points to the mucoprotein components as the active agents. Tamm & Horsfall (55) have recently isolated from

human urine a mucoprotein which is homogeneous both in the electrophoretor and the analytical ultracentrifuge and is an active inhibitor of heated LEE virus. This finding has been confirmed in our laboratory (56). The urinary inhibitor is an unusual type of mucoprotein which is readily soluble in distilled water but becomes less and less soluble as salt concentration increases. The inhibitor in egg-white on the other hand can be partially purified by dilution in a large volume of distilled water when it is precipitated in the ovomucin fraction (48). It has recently been shown that electrophoretically homogeneous mucoprotein from human serum is a highly active inhibitor of indicator virus (57). It appears that the inhibitor quality may be found in a wide variety of mucoproteins and mucoids and that it depends on the presence of certain "functional groups" which can be split off from the macromolecule concerned with relatively little change in its physical properties.

The chemical nature of the interaction between influenza viruses [LEE (B) or MELB (A)] and the two substrates, ovomucin and urinary inhibitor, is under active study in our laboratory and preliminary results have been published (58, 59). When the reaction has proceeded to more than 90 per cent destruction of inhibitor activity, it is found that a dialysable "split-product" can be obtained with N-content equivalent in the case of the urinary inhibitor to about 1 per cent of that of the substrate. The split-product has been partially identified by Gottschalk as an N-substituted isohexosamine (59). The amino acid components are not yet characterized. The residual nondialysable material shows no obvious change in viscosity and is still homogeneous in the electrophoretor but its rate of migration is diminished indicating a decreased negative charge (60). The split-product from virus action on ovomucin is similar to that obtained from urinary inhibitor.

The limited work that has been done on the chemical and physical characteristics of the blood cell surface receptor has left the position rather obscure. De Burgh *et al.* (41) obtained a final product containing carbohydrate and nitrogen and having very high inhibitory power. Striking changes in the reactivity of red cells can be produced by treatment of the cells with very dilute periodate solutions (61, 62). The first effect is to render virus cell union irreversible; i.e., enzymic action cannot occur. With larger amounts of periodate the cells become incapable of adsorbing virus. This of course points strongly toward the likelihood that the specific configuration of certain carbohydrate units is responsible for adsorption of the virus and for susceptibility to enzyme action. Chemically purified but not electrophoretically homogeneous blood group substance from human ovarian cysts had a high inhibitor titre (45) but this could be destroyed by RDE without loss of serological reactivity. Finally it has been shown that after treatment of human erythrocytes with viruses of the influenza group or with RDE there is a sharp change in the electrophoretic velocity, representing a diminution in the net negative charge (63, 64).

In very broad terms it is probably correct to state that both soluble and cell-surface substrates are in the form of macromolecular carbohydrate amino acid compounds (mucoids or mucoproteins). The action of the enzyme is not to break up the macromolecules but to split off functional groups of which there are several to each macromolecule. According to Gottschalk (59), these functional groups are probably N-substituted isohexosamine units attached to a hexose unit of the main part of the molecule. The functional units may well be chemically identical but they differ in their accessibility to enzymic attack. These differences are responsible for the "receptor gradient" (38, 65) and the "inhibitor gradient" (66, 67) shown with red cells and soluble inhibitors respectively.

Adsorption of virus to the red cell surface.—In physiological saline there is rapid adsorption of virus to red cells (7). This adsorption can be prevented in three ways: (*a*) by treatment of the cells with a virus further to the right in the receptor gradient than the virus being tested or with RDE (38), (*b*) by treating the cells with relatively large amounts of periodate (62), and (*c*) by bringing virus and cells together in a solution of very low ionic concentration (68, 69). For adsorption to occur then it is necessary: (*a*) that the functional groups previously referred to should be present, (*b*) that their carbohydrate moieties should not have undergone extensive modification, and (*c*) that appropriate cations should be present in the medium.

Work in our laboratory (69) has fully confirmed earlier work (63), in showing that virus adsorption does not take place when dialysed virus is added to a suspension of fowl cells in isotonic glucose solution free from salts. The cation concentration necessary varies with the virus and of the four "physiological" cations a lower concentration of Ca or Mg is required than of Na or K. It also varies strikingly with the state of the virus, being higher for active virus than for the same virus in its indicator phase.

A virus is said to be converted into the indicator phase when its haemagglutinating action can be inhibited by a typical soluble mucoid inhibitor in high dilution. Influenza B (42) and a few influenza A strains can be so changed by simply heating allantoic fluid virus for 30 minutes at a temperature between 50° and 56°. Many A strains require to be heated in the presence of citrate at an alkaline reaction for the indicator state to be produced (70). In our experience the two requirements for an indicator virus are that it shall still be capable of actively agglutinating red cells and that under the conditions of the test it shall have no enzymic action on the soluble inhibitor being studied (71). Occasional exceptions to this rule have been mentioned in the literature (72), but we should expect that close study of these apparent exceptions will show the normal correlation to exist. Stone's (70) paper contains some good examples of the type of study required. Her demonstration that in some viruses (one strain of PR8 and WSE) a reversible indicator phase could be produced is particularly cogent.

Within the mumps-influenza-Newcastle disease group of viruses, five

different types of union to the red-cell surface can be experimentally demonstrated:

(*a*) Spontaneously reversible union of active virus to the cell surface. Recent work on the progressive change in electrophoretic mobility of human red cells in the presence of relatively small amounts of some active influenza viruses (73) has shown that there is a phase when individual cells show wide variations in their electrophoretic mobility (EPM). This heterogeneity is not seen when the course of RDE action is followed by the same technique. The interpretation offered is that under favourable circumstances a single virus particle may remain attached to a cell for considerable periods and, as it were, "browse" its way over the surface receptors. This finding, taken with the other characteristics of the virus-cell interaction, has some important implications for the mechanism involved. It seems that we must picture the virus particle as carrying a considerable number of enzymically active groups distributed over its surface and similarly that there is a close mesh of mucopolysaccharide receptors over the cell surface. Further, there must be some adsorptive force capable of holding the virus particle in apposition to the cell surface despite the progressive enzymic destruction of the functional groups of the receptor molecules.

It is generally accepted that haemagglutination is a direct result of adsorption of virus particles simultaneously to the surfaces of two erythrocytes so that clumps of cells develop held together by virus bridges. The characteristic thin granular shield obtained on the bottom of the tube suggests that adsorption of virus particles to the surface of the glass may also play a minor part.

(*b*) Reversible union of indicator virus to the cell surface. Indicator virus does not elute spontaneously from red cells but they can readily be stabilized by immune serum or RDE. Provided the reactions are carried out at room temperature or in the cold, such stabilized cells show no evidence of attached virus. Some indicator viruses but not others can be removed from red cells by the addition of a soluble inhibitor (74). Apart from the absence of spontaneous elution, the reactions of indicator viruses are very similar to those of the corresponding active form. If inhibitor is removed from the immune serum by RDE treatment, they can be used for antihaemagglutinin titrations. Ions are necessary for agglutination to occur (69). Stone (70) showed that the position of an indicator virus in the receptor gradient is close to that of its active phase. There is, however, a significant shift to the right; i.e., cells which have been treated with RDE so that they are just not agglutinated by the active phase will be agglutinated by indicator virus. The difference is hardly appreciable with some (MEL) but quite extensive with others (LEE).

(*c*) Irreversible union of active mumps or Newcastle disease viruses. When red cells are treated with either of these viruses and allowed to stabilize, a considerable proportion of the virus initially adsorbed is eluted. If the cells are now washed they produce stable suspensions, but the pres-

ence of irreversibly attached virus on the surface can be demonstrated in three ways: (*a*) The cells are specifically agglutinated by an appropriate antivirus serum (15). (*b*) When mixed with a moderate excess of normal red cells the mixture agglutinates (17). (*c*) Electron micrographs show attached virus particles (75). Treatment with RDE does not liberate the virus (17). With the Australian strain of NDV, this irreversible adsorption does not take place at temperatures below 20°C. Since the haemolytic action of this strain is similarly dependent on temperature, the two phenomena may be related (21).

(*d*) Irreversible union of indicator virus. If fowl cells are treated with indicator virus at 37° for 30 min. or longer and stabilized by treatment with RDE, it will be found that they have irreversibly absorbed the virus (76). In our experience dialysed indicator virus is more effective than crude allantoic fluid virus in inducing this type of reaction. In addition to the capacity to agglutinate normal cells and to be agglutinated by appropriate immune serum, such coated cells are agglutinated to high titre by soluble mucoid inhibitors. The capacity to give this type of irreversible union is strictly correlated with the indicator state.

(*e*) Irreversible union of virus to cells treated with a "modifying" dose of periodate. When fowl or human red cells are treated with periodate solution in the range 0.1 to 2.0 mg. KIO_4 per cc. of packed cells, the cells remain agglutinable by influenza viruses but the adsorbed virus does not elute spontaneously and is not removable by RDE. If the cells are stabilized by the latter reagent and washed they behave similarly to cells on which NDV has been irreversibly adsorbed in being agglutinable by specific immune sera (62). These "PVR" (periodate-virus-RDE) cells are also agglutinated by soluble inhibitors and in fact provide a very convenient means of titrating appropriate inhibitors (69). Perhaps the most striking common feature of all these types of union is that none are possible on cells previously treated with RDE. Such cells neither adsorb nor are haemolysed by NDV (17, 21) and if they are treated with periodate no capacity to adsorb virus develops (62). The "functional groups" which are destroyed by RDE are essential for any of these types of adsorption.

Modification of the cell surface by virus action.-When red cells are treated with the specific enzyme either in the form of active virus or RDE, there is a characteristic sequence of changes demonstrable by three techniques. The cells show minor but definite changes in their immunological reactivity (77), they lose capacity to be agglutinated by viruses of the mumps-influenza-Newcastle disease group (38, 78), and they show diminished electrophoretic mobility (63, 64).

The observation that cells treated with an influenza virus and partially stabilized could be fully stabilized by very dilute immune serum but were strongly agglutinated by lower dilutions of serum (79) was an important step in bringing the action of virus on the cell into line with the Thomsen-Friedenrich (80) phenomenon of panagglutination. Under the influence of

either virus or RDE, cells which were originally stable in any concentration of serum are agglutinated by practically all mammalian sera. The agglutinin titre varies with the serum and with the extent to which the cells have been treated. Fowl cells treated with strain WS gave serum titres around 20, with swine influenza virus around 100, and with the influenza B strain MIL B about 300 (38). The agent responsible can be specifically adsorbed from serum by treated red cells and is generally referred to as "T" agglutinin. Lind & MacArthur (81) made an extensive survey of human sera using human cells treated with RDE for the test suspension. Normal adult sera showed a mean titre of 83, infants under six months (including umbilical and sera) had no T agglutinin and children from one-half to two years had a mean titre of 24. Immunization of rabbits showed that RDE-treated cells had a new antigenic character not manifested by normal cells (77). Sera from cases of atypical pneumonia showed an abnormally high T agglutinin content (81).

The receptor gradient.—The first demonstration that treatment with one type of virus did not necessarily result in the loss of agglutinability for other viruses was made with cells which had been stabilized after the action of NDV (14). These are normally agglutinated by influenza viruses. Detailed study of these reactions has given rise to the concept of a receptor gradient by which viruses can be arranged in a linear order, those earlier in the gradient being unable to agglutinate partially modified cells still capable of agglutination by viruses later in the gradient (38). The order depends both on the species providing the red cell and the agent used to modify the cells. The most convenient way of demonstrating the phenomenon is to use cells treated for a standard time with graded dilutions of RDE. The action is stopped by the addition of citrate and the washed cells are then tested against the whole series of viruses in citrate saline.

The degree to which viruses can reduce the agglutinability of cells depends on the concentration of the virus and the time of contact. In our own earlier work, cells were tested after a few hours contact with allantoic fluid virus and stabilized if necessary with dilute immune serum. Under these circumstances loss of receptors proceeded only so far in the sequence as was necessary to render the cells inagglutinable by the virus being used. With concentrated virus, Hirst (78) was able to push the destruction of receptors much further. In their work on changes in EPM referred to in the next paragraph, Ada & Stone (64) also found it possible to produce more extensive effects by prolonged treatment with virus.

Changes in electrophoretic mobility.—The change in EPM was first demonstrated by Hanig (63), but the most extensive studies are those of Ada & Stone (64). Their findings are complex and not easily abstracted. In general the susceptibility to agglutination by viruses at different points on the receptor gradient is lost at definite levels of EPM. Some viruses however, notably NDV, SW, and mumps can under special conditions depress the EPM well beyond the level associated with loss of agglutina-

bility by these viruses. It appears that some of the functional groups (substrate) on the cell surface play only a minor part in determining virus adsorption although contributing equally to the net negative charge (82). No virus reduces the EPM to the level obtained with RDE (0.17 μ). The point at which human cells treated with the appropriate agents become inagglutinable by a given virus does not necessarily correspond to a constant level of EPM. This can be shown by a table constructed from data in Stone & Ada's paper (83).

TABLE 1

CHANGES IN EPM UNDER ACTION OF VIRUSES AND RDE

Cells treated with	EPM at agglutinability end point for								
	MU	NDV	MEL	PR8	BEL	LEE	SW	MIL.B	Full action
MEL	1.19	1.05	0.96	0.92					
NDV	0.80	0.76	0.73	0.55	0.46				
LEE	1.02	1.01	1.01	0.93	0.82				0.72
RDE	0.44	0.81	0.74	0.65	0.39	0.39	0.39	0.25	0.17

Ada & Stone have deduced that there are two types of substrate A and B which when destroyed or modified reduce the average EPM. Most viruses act only on the A series, but NDV and SW as well as RDE have an action on the B series as well.

Interpretation of the virus-cell surface interaction.—There is no uniformity of interpretation of these phenomena and it is likely that their final interpretation will require a greater knowledge of the nature of enzyme action than is at present available. Nevertheless, the reviewer has for some time contended (53, 54) that the influenza red cell system has some uniquely favourable attributes for the study of enzyme-substrate action when both reagents are incorporated in the surface of relatively massive carriers, and that it is as justifiable to look for fundamental characteristics of enyzme action as manifested in this system as to wait for developments in more orthodox experimental systems to provide a basis for its interpretation.

It is now universally accepted that virus particles themselves are the haemagglutinating agents and that adsorptive groups and enzymically active groups (irrespective of whether they are the same or different) are integral parts of the virus surface. Everything points to the essential identity of the virus action on soluble inhibitors (mucoproteins) and on the cell surface.

A simplified picture of the process which has emerged from discussions in this laboratory is as follows:

The interactions are dominated by the reaction between enzyme and

substrate as modified by the fact that both these entities form parts of larger surfaces so that immediately adjacent atomic groupings, as well as enzyme and substrate in the limited sense, can play a significant part. Adsorption of virus to substrate carrier takes place in at least two stages, possibly more. The primary phase involves, in the case of virus-cell union, the development of multiple adsorptive bonds and is markedly influenced by the ionic environment, Ca^{++} being particularly effective. Both features suggest that electrostatic forces are mainly concerned and give rise essentially to an unorientated apposition of enzyme and substrate groupings. Van der Waal's type forces arising from the interaction of groupings adjacent to the specific enzyme and substrate groups respectively probably play the major part in determining the phenomena of the receptor gradient. With an active virus particle, this primary adsorption plus random thermal agitation allows progressive opportunities for mutual orientation of enzyme and substrate until activation and destruction of substrate occurs with elimination of a major adsorptive bond. The development of mutual orientation and chemical reaction is regarded as the second phase of the adsorptive process. It may be followed either by release of the virus particle from the cell surface or by the development of a new adsorptive situation without release so that further enzymic destruction of substrate can take place and allow the browsing phenomenon described earlier (66) to occur. Under appropriate circumstances, orientated enzyme-substrate reaction instead of provoking typical substrate destruction may lead to a stable chemical union (analogous to competitive inhibition of an enzyme by a molecule related to the normal substrate). Such union is postulated to account for the phenomena: (*a*) of irreversible attachment of mumps and NDV to red cells, (*b*) of irreversible union of indicator virus to fowl cells (76), and (*c*) of any type of influenza virus to periodate-treated cells. It should be made clear that these secondary processes of chemical union resulting either in the enzymic destruction of substrate or in the formation of a stable compound play no significant part in the normal adsorptive and haemagglutinating reactions which result from the primary stage of adsorption.

Reaction of virus with soluble inhibitor probably follows similar stages modified by the likelihood that except in the case of urinary mucoprotein, fewer adsorptive bonds develop between virus and macromolecule than between virus and cell surface. The occurrence of irreversible union between indicator virus and inhibitor has not been demonstrated but is strongly suggested by the progressive increase in inhibition that results from continuing contact of virus with inhibitor.

There is still no satisfactory interpretation of the indicator state. The simplest way of looking at the change is that indicator virus tends to remain attached to the substrate with which it first makes contact, whereas active virus is more likely to reach an equilibrium as between soluble inhibitor and cell surface with the advantage strongly on the side of the cell surface. Possibly the most illuminating experiments are those with urinary mucopro-

tein as inhibitor (84). Unlike other inhibitors this is highly active against MEL virus to whose enzyme it is hardly susceptible, while LEE, which has a potent enzymic action, is not inhibited in the active state. When PVR cells are used, MEL-coated cells are agglutinated to almost the same titres as those coated with the indicators T.MEL and H.LEE. All the other inhibitors that have been tested fail to agglutinate PVR cells with active (nonindicator) viruses. This fact strongly indicates that an essential part is played by the absence of enzyme in determining the indicator state.

In the case of strain LEE, no evidence has been published or observed in this laboratory that the indicator state can be produced without loss of enzymic activity. With MEL and PR8 it has been found that by heat treatment under graded conditions there is an intermediate zone in which the strict negative correlation between indicator state and enzymic activity breaks down (71). It has also been shown that chemical agents such as sodium hydrosulphide (85) and ether (72) may produce indicator virus with some enzymic action but only very close to the point at which haemagglutinin is destroyed.

The reviewer's opinion which, however, is not accepted by some of his colleagues (85), is that the inhibition of indicator virus as against active virus is based on three points: (*a*) In the indicator virus the (inactive) enzyme group retains its essential structure so that it can react with substrate both by primary adsorption and by secondary union to form a stable compound; (*b*) the absence of enzymic action eliminates one important means by which union of the carriers of enzyme and substrate can be broken; and (*c*) the number of adsorptive bonds developed in the primary stage is greater for virus-cell than for virus-inhibitor union.

None of the reactions can occur if substrate is destroyed by prior treatment with RDE or an appropriately active virus. Enzyme-substrate reaction must therefore play a dominant part in all, but the almost infinite variety of results obtained with different viruses, cells, and inhibitors speaks equally strongly for the importance of modifying factors other than the specific groups of enzyme and substrate.

The Reaction of Influenza Virus with the Surface of Susceptible Cells

The interactions of viruses with red cells are only important for the understanding of virus disease insofar as they provide models of what takes place in the process of infection. The first demonstration that adsorption and elution of virus could take place on the surface of susceptible respiratory cells was given by Hirst (8) using the excised ferret lung. He also showed that the mouse lung could be used for the same type of experiment and this system has been extensively exploited by Fazekas de St. Groth (86). It is easy to show that when relatively large amounts of influenza virus are "inspired" into the trachea and bronchial system of the excised mouse-lung preparation, some time elapses before it can be recovered in

saline washings, but it is then eluted in similar fashion to the release from red cells, 50 per cent being recoverable within 2 hr. from the initial instillation. Prior treatment with RDE renders the excised lung incapable of absorbing virus. Mice can be treated with RDE *in vivo* and after appropriate dosage subsequent tests on the excised lung shows characteristic diminution in the power to absorb virus. If this is taken as a measure of the cell surface receptors present it can be shown that regeneration of receptors after RDE destruction begins in about 8 hr. from the time the RDE is removed and rapidly re-establishes the normal state. It was not possible to exhaust the capacity for regeneration by prolonged RDE treatment of mice (87).

The most direct evidence of the importance of cell receptors is afforded by experiments on the prevention of infection by treatment of the susceptible cells with RDE (88, 89, 90). A significant effect can be shown in the allantoic cavity of the chick embryo, in the mouse lung and against the neurotropic influenza virus strain NWS in the mouse brain. Stone (88) found that in chick embryos RDE treatment was much more effective in preventing infection by some strains than by others and gave evidence for believing that differences exist in the requirements in terms of receptor concentration for infection with different strains. In general recently isolated strains were more readily prevented from multiplication than strains long adapted to allantoic passage.

Similar results were obtained in mice (89), again with marked differences in the degree of protection obtained against different strains. There was a distinct suggestion, in Stone's experiments that strains early in the receptor gradient, MEL, WS and PR8, required a higher concentration of cell-surface receptors to allow initiation of infection than strains like LEE and SW, which come later in the gradient. In both chick embryos and mice, rapid regeneration of receptors occurs and no serious consideration has been given to the possibility of developing the principle of protection by removal of receptors to a practical level.

In Cairns (90) experiments using highly purified RDE in large dose, the interesting feature in addition to the protective effect on subsequent challenge was the complete destruction of inhibitor in all parts of the brain following a small local injection of RDE in one hemisphere.

Further analysis of the process of infection in chick embryos has been carried out by the use of RDE at different stages. The enzyme has the valuable property of releasing virus bound to the surface of the cell and allowing its subsequent titration by haemagglutination in the presence of citrate. In embryos killed by formalin, virus is taken up and spontaneously elutes in 1 to 3 hr. (88). At any stage it can be released completely by RDE (91). In living embryos no elution is detectable before the first release of newly formed virus. The amount of virus that can be released by RDE diminishes progressively for 2½ to 3 hr. (91), presumably on account of its entry into the cell substance.

The influence of periodate on receptor substance, allowing irreversible

union with virus, can be clearly shown in the mouse lung (92). Virus cannot be released by RDE action in excised lungs treated with periodate. Mice given periodate intranasally an hour before challenge with graded virus dilutions show normal susceptibility to infection but they cannot be protected by the inclusion of RDE in the virus inoculum. Several types of experiment on living chick embryos treated with periodate also gave decisive evidence that although union with cell-surface receptors is an essential step in infection, subsequent enzymic liberation from that union is not needed. Fazekas de St. Groth (91) has named the process by which a virus particle held to the cell surface by adsorption to mucoid receptors is gradually taken into the cell *viropexis*, by analogy with the process of colloidopexis.

No information is available as to whether cell receptors play any part in the "toxic" effect of influenza virus (93, 93a). It has been suggested that the pyrogenic action of influenza virus in rabbits shows evidence of a gradient effect (94), but unpublished experiments in this laboratory (95) have not confirmed the claim that pretreatment with RDE abolished the thermal response.

The Biological Significance of the Phenomena

Influenza virus infections in nature involve cells exposed to the air in the respiratory passages but in part protected by a film of mucin which is steadily moved toward the upper respiratory tract by ciliary action. On the free surface of the cells there is histochemical evidence of polysaccharide-containing material. It is natural therefore to look at the adsorptive-enzymic properties of the influenza viruses as having evolved in relation to these aspects of their natural environment.

Fazekas de St. Groth (96) finds, in the course of experimental infection in mice, that there is a reproducible sequence of destruction of free inhibitor and of fixed receptors in the lung tissue followed, as the infection is overcome, by their reappearance. In the acute stages of human influenza, nasal secretion shows a changed inhibitor quality which probably represents partial modification of the mucoids concerned by the enzymic action of the infecting strain (97).

Not very much has been published in regard to the properties of human bronchial mucus as a virus inhibitor mainly because of the difficulties of handling such material. It is known to have typical inhibitory effects on haemagglutination by indicator viruses and to a lesser extent on some active viruses, and capacity to inhibit infection in mice has been briefly reported (98).

If we accept the view that enzymic destruction of receptors is not a necessary part of the initiation of cell infection, it seems necessary to look at the function of the enzyme as a means of liberating itself from adsorbed mucins which would otherwise prevent its making effective contact with the susceptible host cell surface. Some suggestions as to the part mucin may play in clinical influenza are made in a paper by Himmelweit (99).

Vaccinia and Related Haemagglutinins

Washed vaccinia virus particles have some haemagglutinating power (100) but most of the haemagglutinin in a typical chorioallantoic membrane extract is easily shown to be distinct from the virus. Filtration through gradocol membranes (101) indicates that it is in the form of relatively large units about 65 mμ in diameter. Similar haemagglutinins are produced by ectromelia (29) and variola (102) viruses.

Vaccinia haemagglutinin is demonstrable only on chicken and pigeon cells and only on the cells of a proportion of birds (103, 104). Cells from chick embryos or day-old chicks are unaffected but some chickens give susceptible cells at 14 days of age and the normal proportion (40 to 60 per cent) is attained at 28 days (98). The same behaviour of chicken cells is shown to agglutination by lipid extracts of a variety of normal tissues as well as with purified lecithin-cholesterol dispersions in saline (103). Lipid extracts also agglutinate susceptible pigeon cells and all mouse cells. Ectromelia agglutinins, in addition to acting on the same fowl and pigeon cells, agglutinate mouse cells which are not agglutinated by vaccinia virus preparations (29, 104).

Virus agglutinins of this group are inhibited by appropriate immune sera but not by normal sera. Lipid haemagglutination is readily inhibited by any serum (103). Collier (105) has described an inhibitor of vaccinia haemagglutination that can be extracted from normal mammalian lungs. The hypothesis that vaccinia and ectromelia haemagglutinins were essentially complexes of an antigenic virus component with a phospholipid was virtually established by Stone's demonstration that two different types of lecithinase (*Cl. welchii* toxin α and cobra venom) destroyed the haemagglutinins (106).

No spontaneous elution of adsorbed haemagglutinin occurs, but the agglutinin can be removed by immune serum and after washing the cells are found to be susceptible to reagglutination by vaccinia haemagglutinin. Cells treated by RDE show no change in their reactivity (104). Fisher (107) found that protamine and vaccinia haemagglutinin mutually destroy their agglutinating capacities for susceptible fowl cells and has suggested that the cell receptor concerned is a basic protein.

Much use has been made of the haemagglutinin reaction in immunological studies of smallpox and vaccinia infections and in experimental studies of mousepox, but these have not contributed significantly to an understanding of the nature of the reaction.

Pneumonia Virus of Mice

Haemagglutination by this virus (PVM) was first described by Mills & Dochez (25), but detailed study of the phenomena has come almost wholly from Horsfall's laboratory (26, 68, 108). As in the case of the influenza

virus group, the haemagglutinating agent is the virus particle itself. Free virus particles in the infective state can only be obtained when special precautions are taken to prevent union with some tissue component of mouse lungs. As ordinarily obtained by grinding infected mouse lungs with saline, virus suspensions have no haemagglutinating power, but if the suspension is made in a solution of low electrolyte content it is both infective and haemagglutinating. The haemagglutinating and antigenic properties of the virus are highly heat resistant and in the earlier stages of the work haemagglutinating preparations were made by heating saline emulsions of infected mouse lungs at 80°C. for 10 minutes. This degree of heating is sufficient to destroy the inhibitory agent and release the virus particles.

The only species which provide cells susceptible to agglutination by PVM are mouse and hamster. Virus is firmly adsorbed to the cells and there is no evidence of any spontaneous elution. It can however be released as from tissue emulsions by heating to 80°. Everything points to the likelihood that the virus is adsorbed to the same component in red cells and tissue cells of mouse and hamster and that a similar agent is present in cotton rat lung though not in erythrocytes of that species.

Beyond the fact of its heat-lability nothing is known of the nature of the host component. Ginsberg (109) assumes that it is of protein character but no report of its isolation has appeared. Its absence from tissues of the chick embryo, in which no multiplication of the virus occurs, plus its presence in high activity only in tissues susceptible to infection, suggests strongly that the agent plays a significant part in the process of cell infection.

LITERATURE CITED

1. Burnet, F. M., *Brit. J. Exptl. Path.*, **21,** 147 (1940)
2. Nigg, C., Crowley, J. H., and Wilson, D. E., *Science*, **91,** 603 (1940)
3. Burnet, F. M., *Australian J. Exptl. Biol. Med. Sci.*, **19,** 291 (1941)
4. Hirst, G. K., *Science*, **94,** 22 (1941)
5. McClelland, L., and Hare, R., *Can. J. Pub. Health*, **32,** 530 (1941)
6. Hirst, G. K., *J. Exptl. Med.*, **75,** 49 (1942)
7. Hirst, G. K., *J. Exptl. Med.*, **76,** 195 (1942)
8. Hirst, G. K., *J. Exptl. Med.*, **78,** 99 (1943)
9. Burnet, F. M., Beveridge, W. I. B., Bull, D. R., and Clark, E., *Med. J. Australia*, **II,** 371 (1942)
10. Salk, J. E., *J. Immunol.*, **49,** 87 (1944)
11. Burnet, F. M., and Bull, D. R., *Australian J. Exptl. Biol. Med. Sci.*, **21,** 55 (1943)
12. Levens, J. H., and Enders, J. F., *Science*, **102,** 117 (1945),
13. Lush, D., *J. Comp. Path. Therap.*, **53,** 157 (1943)
14. Burnet, F. M., *Australian J. Exptl. Biol. Med. Sci.*, **20,** 81 (1942)
15. Burnet, F. M., and Anderson, S. G., *Brit. J. Exptl. Path.*, **27,** 236 (1946)
16. Burnet, F. M., *Brit. J. Exptl. Path.*, **27,** 244 (1946)
17. Anderson, S. G., *Australian J. Exptl. Biol. Med. Sci.*, **25,** 163 (1947)
18. Morgan, H. R., Enders, J. F., and Wagley, P. F., *J. Exptl. Med.*, **88,** 503 (1948)

19. Chu, L. W., and Morgan, H. R., *J. Exptl. Med.*, **91,** 393, 403 (1950)
20. Kilham, L., *Proc. Soc. Exptl. Biol. Med.*, **71,** 63 (1949)
21. Burnet, F. M., and Lind, P. E., *Australian J. Exptl. Biol. Med. Sci.*, **28,** 129 (1950)
22. Taylor, R. M., *Am. J. Pub. Health*, **39,** 171 (1949)
23. Francis, T., Quilligan, J. J., and Minuse, E., *Science*, **112,** 495 (1950)
24. Hirst, G. K., *J. Exptl. Med.*, **91,** 177 (1950)
25. Mills, K. G., and Dochez, A. R., *Proc. Soc. Exptl. Biol. Med.*, **57,** 140 (1944)
26. Curnen, E. C., and Horsfall, F. L., Jr., *J. Exptl. Med.*, **83,** 105 (1946)
27. Nagler, F. P. O., *Med. J. Australia*, **I,** 281 (1942)
28. Burnet, F. M., *Nature*, **155,** 543 (1945)
29. Burnet, F. M., and Boake, W. C., *J. Immunol.*, **53,** 1 (1946)
30. Hallauer, C., *Schweiz. Z. Path. u. Bakt.*, **9,** 553 (1946)
31. Olitsky, P. K., and Yager, R. H., *Proc. Soc. Exptl. Biol. Med.*, **71,** 719 (1949)
32. Verlinde, J. D., and De Baan, P., *Ann. inst. Pasteur*, **77,** 632 (1949)
33. Jungleblut, C. W., *Bull. N. Y. Acad. Med.*, **26,** 571 (1950)
34. Lahelle, O., and Horsfall, F. L., *Proc. Soc. Exptl. Biol. Med.*, **71,** 713 (1949)
35. Sabin, A. B., and Buescher, E. L., *Proc. Soc. Exptl. Biol. Med.*, **74,** 222 (1950)
36. Sabin, A. B., *Federation Proc.*, **10,** 573 (1951)
37. McCrea, J. F., *Australian J. Exptl. Biol. Med. Sci.*, **25,** 127 (1947),
38. Burnet, F. M., McCrea, J. F., and Stone, J. D., *Brit. J. Exptl. Path.*, **27,** 228 (1946)
39. Burnet, F. M., and Stone, J. D., *Australian J. Exptl. Biol. Med. Sci.*, **25,** 227 (1947)
40. Ada, G. L., and French, E. L., *Australian J. Sci.*, **13,** 82 (1950)
41. De Burgh, P. M., Yu, P. C., Howe, C., and Bovarnick, M., *J. Exptl. Med.*, **87,** 1 (1948)
42. Francis, T., *J. Exptl. Med.*, **85,** 1 (1947)
43. Briody, B. A., *J. Immunol.*, **59,** 115 (1948)
44. Anderson, S. G., *Australian J. Exptl. Biol. Med. Sci.*, **26,** 347 (1948)
45. Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Nature*, **160,** 404 (1947)
46. Burnet, F. M., *Australian J. Exptl. Biol. Med. Sci.*, **26,** 371 (1948)
47. French, E. L., and Curtain, C. C., *Australian J. Exptl. Biol. Med. Sci.* (In press)
48. Gottschalk, A., and Lind, P. E., *Brit. J. Exptl. Path.*, **30,** 85 (1949)
49. Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *J. Biol. Chem.*, **179,** 1275 (1949)
50. Anderson, S. G., *Australian J. Science*, **12,** 147 (1950)
51. McCrea, J. F. (In preparation)
52. Anderson, S. G., Burnet, F. M., Fazekas de St. Groth, S., McCrea, J. F., and Stone, J. D., *Australian J. Exptl. Biol. Med. Sci.*, **26,** 403 (1948)
53. Burnet, F. M., *Proc. Roy. Soc.* (*London*), [B] **138,** 47 (1951)
54. Burnet, F. M., *Physiol. Revs.*, **31,** 131 (1951)
55. Tamm, I., and Horsfall, F. L., Jr., *Proc. Soc. Exptl. Biol. Med.*, **74,** 108 (1950)
56. Ada, G. L., and Gottschalk, A. (In preparation)
57. Stulberg, C. S., Schapira, R., Robinson, A. R., Basinski, D. H., and Freund, H. A., *Proc. Soc. Exptl. Biol. Med.*, **76,** 704 (1951)
58. Gottschalk, A., and Lind, P. E., *Nature*, **164,** 232 (1949)
59. Gottschalk, A., *Nature*, **167,** 845 (1951)

60. Ada, G. L., and Pye, J. (Unpublished data)
61. Hirst, G. K., *J. Exptl. Med.*, **87**, 301 (1948)
62. Fazekas de St. Groth, S., *Australian J. Exptl. Biol. Med. Sci.*, **27**, 65 (1949)
63. Hanig, M., *Proc. Soc. Exptl. Biol. Med.*, **68**, 385 (1948)
64. Ada, G. L., and Stone, J. D., *Brit. J. Exptl. Path.*, **31**, 263 (1950)
65. Burnet, F. M., *Australian J. Exptl. Biol. Med., Sci.*, **23**, 180 (1945)
66. Stone, J. D., *Australian J. Exptl. Biol. Med. Sci.*, **27**, 557 (1949)
67. Hirst, G. K., *J. Exptl. Med.*, **91**, 161 (1950)
68. Davenport, F. M., and Horsfall, F. L., *J. Exptl. Med.*, **88**, 621 (1948)
69. Burnet, F. M., and Edney, M., *Australian J. Exptl. Biol. Med. Sci.*, **30**, 105 (1952)
70. Stone, J. D., *Australian J. Exptl. Biol. Med. Sci.*, **27**, 337 (1949)
71. Edney, M., and Burnet, F. M., *Australian J. Exptl. Biol. Med. Sci.*, **30**, 129 (1952)
72. Smith, W., and Westwood, J. C. N., *Brit. J. Exptl. Path.*, **31**, 725 (1951)
73. Ada, G. L., and Stone, J. D., *Brit. J. Exptl. Path.* (In press)
74. Burnet, F. M. (Unpublished data)
75. Burnet, F. M., and Mitchell, R. F. (Unpublished data)
76. Burnet, F. M., *Australian J. Exptl. Biol. Med. Sci.*, **30**, 119 (1952)
77. Burnet, F. M., and Anderson, S. G., *Australian J. Exptl. Biol. Med. Sci.*, **25**, 213 (1947)
78. Hirst, G. K., *J. Exptl. Med.*, **91**, 161 (1950)
79. Burnet, F. M., *Lancet*, **I**, 7 (1948),
80. Friedenreich, V., *Acta Path. Microbiol. Scand.*, **5**, 59 (1928)
81. Lind, P. E., and McArthur, N. R., *Australian J. Exptl. Biol. Med. Sci.*, **25**, 247 (1947)
82. Stone, J. D., and Ada, G. L., *Brit. J. Exptl. Path.*, **31**, 275 (1950)
83. Stone, J. D., and Ada, G. L., *Brit. J. Exptl. Path.* (In press)
84. Burnet, F. M., *Australian J. Exptl. Biol. Med. Sci.* (In preparation)
85. Stone, J. D., and Fazekas de St. Groth, S. (Unpublished data)
86. Fazekas de St. Groth, S., *Australian J. Exptl. Biol. Med. Sci.*, **26**, 29 (1948)
87. Fazekas de St. Groth, S., *Australian J. Exptl. Biol. Med. Sci.*, **26**, 271 (1948)
88. Stone, J. D., *Australian J. Exptl. Biol. Med. Sci.*, **26**, 49 (1948)
89. Stone, J. D., *Australian J. Exptl. Biol. Med. Sci.*, **26**, 287 (1948)
90. Cairns, H. J. F., *Nature*, **168**, 335 (1951),
91. Fazekas de St. Groth, S., *Nature*, **162**, 294 (1948)
92. Fazekas de St. Groth, S., and Graham, D. M., *Australian J. Exptl. Biol. Med. Sci.*, **27**, 83 (1949)
93. Henle, G., and Henle, W., *J. Exptl. Med.*, **84**, 623 (1946)
93a. Henle, W., and Henle, G., *J. Exptl. Med.* **84**, 639 (1946)
94. Wagner, R. R., and Bennett, I. L., *J. Exptl. Med.*, **91**, 135 (1950)
95. French, E. L., *Australian J. Exptl. Biol. Med. Sci.* (In press)
96. Fazekas de St. Groth, S., *Lancet*, **I**, 1101 (1950)
97. Fazekas de St. Groth, S., *Nature*, **167**, 43 (1951)
98. Rose, H., *Federation Proc.*, **9**, 390 (1950)
99. Himmelweit, F., *Trans. Med. Soc. London*, **65**, 438 (1947–49)
100. Gillen, A. L., Burr, M. M., and Nagler, F. P., *J. Immunol.*, **65**, 701 (1950)
101. Chu, C. M., *J. Hyg.*, **46**, 49 (1948)

102. Broben, J. A., and North, E. A., *Med. J. Australia*, **II**, 229 (1943)
103. Stone, J. D., *Australian J. Exptl. Biol. Med. Sci.*, **24**, 197 (1946)
104. Burnet, F. M., and Stone, J. D., *Australian J. Exptl. Biol. Med. Sci.*, **24**, 1 (1946)
105. Collier, W. A., *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **15**, 97 (1949)
106. Stone, J. D., *Australian J. Exptl. Biol. Med. Sci.*, **24**, 191 (1946)
107. Fisher, S., *Australian J. Sci.*, **11**, 27 (1948)
108. Curnen, E. C., Pickels, E. G., and Horsfall, F. L., *J. Exptl. Med.*, **85**, 23 (1947)
109. Ginsberg, H. S., *Federation Proc.*, **10**, 570 (1951)
110. White, F., *Australian J. Sci.* (In press)

INFLUENCE OF AGE ON SUSCEPTIBILITY TO VIRUS INFECTIONS WITH PARTICULAR REFERENCE TO LABORATORY ANIMALS[1]

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In reviewing the literature on the relationship between age and susceptibility to virus infections it became clear that the so-called "age factor" does not represent a single influence but rather a complex interaction of many elements. It was, therefore, felt that an analytic approach would be most suitable for bringing out the components. Accordingly the review is divided into three parts and each part is subdivided into several sections and subsections. Part I depicts the variable outcomes of encounters between viruses and hosts of different ages under natural conditions. Part II illustrates: (*a*) the importance of extrinsic and intrinsic factors in the determination of the changes in susceptibility with maturation; (*b*) the variable manifestations of these changes; and (*c*) the variability of the direction of these changes. Part III attempts a correlation between the changes in susceptibility and the alterations in certain constitutional factors in the course of development and maturation. The review concerns chiefly the changes in susceptibility that occur in the course of embryonal development, early postnatal life, and adult life. We could find very little information on changes in senescence. The following table contains the breakdown of all sections and subsections:

I. Infections under Natural Conditions

- A. Natural Infections in Animals
  - a. Interaction of age, genetic and physiologic factors
  - b. Age differences expressed in a different clinical picture
  - c. Establishment of a latent infection under the influence of the "age factor"
  - d. Infection of the fetus under natural conditions

II. Experimental Infections

- A. Infections of the Embryo
  - a. The guinea pig
  - b. The chick embryo
- B. Changes in the Susceptibility of Chick Embryos in the Course of Their Development as Determined by Different Procedures
  - a. The severity of infection

[1] The survey of the literature pertaining to this review was concluded in March, 1952.

b. Dosage required to induce infection
c. Multiplication of virus
C. Infections of the Postnatal Experimental Animal
a. Differences encountered with different routes of inoculation in mice
b. Differences obtained with different neurotropic viruses and with "fresh" and passaged strains of the same virus
c. The behavior of the neurotropic viruses in animals other than the mouse
d. Determination of age-induced resistance to Theiler's virus in mice from infected and virus-free colonies
D. Alteration of the Clinical Picture as a Manifestation of Age-Induced Resistance
a. The Western equine encephalomyelitis virus
b. The Coxsackie viruses
c. Tumor producing viruses
E. Other Manifestations of Resistance Acquired with Age
F. The Variability of the Direction of Change in Susceptibility with Growth and Development
G. Some Practical Considerations

III. Consideration of the Possible Mechanisms in the Influence of Age on Susceptibility to Virus Infections

A. Fundamental and Special Mechanisms
a. Metabolism
b. Anatomical and cytological changes
c. Localization of virus in skin
d. The hemoencephalitic barrier
e. Antibodies
f. Hormones
g. Inhibitory substances
h. Certain virus factors
B. Regional and "Subtotal" Resistance

IV. An Attempt at Synthesis

The subject matter of this chapter deals with resistance of animals to viruses. It was felt, however, that certain results of studies on plant and bacterial viruses might be pertinent. Working with the tobacco mosaic virus, Holmes (1) noted that young plants of *Solanum melongena* var. Black Beauty were much more severely affected than were older plants. In general, there was better localization of virus in older plants, whereas in younger plants virus spread and caused systemic necrosis. The youngest leaves of old plants appeared to be more resistant than similar leaves of younger plants as demonstrated by some localization of virus in them. Plants were generally killed if inoculated when they were about two inches

high, but when they reached the height of four inches, they could be infected without the development of systemic necrosis.

Similarly, the age of the host plays a very important role in the infection of bacteria with bacteriophage. Spizizen (2) found that more phage multiplied in cells obtained before the onset of the logarithmic phase of bacterial growth. Almost no phage was produced in the stationary phase of *Escherichia coli* growth. In a monograph on bacteriophage T_2, Heden (3) showed that phage yield determined in "single infection—one step experiments" reached a maximum at the transition from the lag to the logarithmic phase of bacterial growth. This was coincident in time with the maximum ribose nucleic acid content and maximum volume of individual cells.

INFECTIONS UNDER NATURAL CONDITIONS

Many factors tend to complicate the evaluation of natural resistance of man. They include such diverse elements as active and passive immunity, climate, economic status, and accuracy of recognition and reporting of disease. For this reason and in view of the restriction of space, the review deals largely with infections of animals. The effect of several viruses on the human fetus will be cited at the end of Part I. The bibliography lists a number of papers which contain information on age incidence, case fatality rate, and other aspects of virus diseases in man (4 to 30). Illuminating discussions on the interaction of extrinsic and intrinsic factors in poliomyelitis are given by Hammon (31) and Sabin (32).

Natural Infections in Animals

Four illustrations were selected to bring out the variability of the effect of age on the outcome of an infection. One exemplifies an interplay of age with physiologic and genetic factors in the host-virus relationship. The second points up an alteration of the clinical picture as a reflection of a change in susceptibility. The third exhibits an infection which leads to a state of latent parasitism. The fourth shows that a relatively innocuous virus can cause severe damage when it finds its way into the tissues of the immature fetus.

Interaction of age, genetic and physiologic factors.—Greene (33) describes the factors which influenced an epidemic of rabbit pox. Age was a most significant element as illustrated by the mortality rate of 71.8 per cent in young animals compared with 14.7 per cent in adults. The highest mortality occurred between the ages of four and nine weeks. This interval is a transitional period in the rabbit's life between strict dependence on maternal care and food and independent existence. The susceptible age was much earlier when the nursing period was forcibly shortened. The apparent protective effect of nursing is strikingly reminiscent of the observations on the 1949 poliomyelitis epidemic among Eskimos (26, 32), who often nurse their children for as long as three years. In this epidemic, only young children escaped illness. The question can therefore be raised as to the possible

induction of a state of passive immunity by milk. The data obtained from the poliomyelitis epidemic suggest, however, that with the exception of the babies, the Eskimo population was highly susceptible. Since, as was pointed out by Sabin (32), the antipoliomyelitic factor of human milk appears to be an antibody, the high degree of susceptibility in the adult population militates against the possibility that the sparing of the children reflected specific immunity passively acquired during nursing. Likewise, although serologic determinations of immunity to rabbit pox were not carried out, Greene's reports (33, 35) offer ample evidence that specific immunity was not responsible for the lower mortality in very young nurslings in the early phases of the epizootic. Thus, the reason for the apparent protective effect of nursing remains obscure.

The interaction of age and heredity is revealed by the analysis of susceptibility of several pure breeds. Thus, the mortality rates for young and adult animals were 68.7 per cent and 0 per cent in Himalayan rabbits, but they were 94.3 and 33 per cent in the more vulnerable Belgian Hare rabbits.

Age differences expressed in a different clinical picture.—Newcastle disease of chickens gives rise to different symptoms in young and adult chickens. As brought out by Beach (36), what were thought to be two separate diseases, one a respiratory—nervous disorder of chicks 2 to 10 weeks of age and the second "chicken flu" or "nine-day pneumonia" in mature chickens, appear to be caused by the same virus. Fabricant (37) has described some of the clinical features of infection with Newcastle disease virus in New York State. Young chicks and broilers exhibited respiratory and neurologic symptoms. In mature chickens, the illness was limited to respiratory symptoms, loss of appetite, and decreased egg production.

Establishment of a latent infection under the influence of the "age factor."—The problem of latent infections is of great significance from the standpoint of evolutionary ecology as well as from the point of view of preventive medicine. It represents a situation which is ideal for the survival of host and parasite—a peaceful coexistence. Herpes simplex in man and Theiler's encephalomyelitis and lymphocytic choriomeningitis in mice are oftenmentioned examples of latent infections. Only lymphocytic choriomeningitis (LCM) will be cited presently. Traub (38, 39, 40, 42) has observed a remarkable evolution in the host-virus relationship culminating in an equilibrium of the reactions of the antagonists. He found (38) that lymphocytic choriomeningitis occurring under natural conditions in a mouse colony was mild, highly contagious, but with low morbidity and very low mortality. In the beginning, symptoms were noted in mice that were infected *in utero* (39), while mice infected by contact soon after birth remained asymptomatic. The age of the animal determined not only susceptibility to infection but also the length of the carrier state following infection (40). Mice infected *in utero* carried virus for longer periods than young mice infected by contact. The development and duration of the carrier states was, however, also dependent on the attributes inherent in strains of virus with different pas-

sage histories. The route of infection with different strains of the virus was another important factor (40). With mouse-passage virus injected nasally, only one-day-old mice died, and mice 12 to 14 days or five weeks old remained well. The natural virus (colony virus) given by the same route caused death in some two-week-old mice and illness in a few five-week-old animals. When the more virulent guinea pig-passaged virus was administered by the same route, all five-week and nine- to ten-week-old mice exhibited clinical evidence of infection. In contrast are the findings of Whitney (41)[2] illustrating the difference in vulnerability of mice of different ages when inoculated with "mouse-passaged" or "wild" strains of lymphocytic choriomeningitis virus by the cerebral route. Both strains had the same lethality for adult mice (about 100 per cent). In baby mice, however, the laboratory strain (mouse-passaged) induced a 75 per cent mortality while strains recently isolated from house mice or human material ("wild" virus) caused a mortality of only 17 per cent. The infant mice, although much less vulnerable than the adults, were nevertheless susceptible to the fresh strains. They developed inapparent infections as evidenced by their becoming resistant to the virus.

Thus, the mortality of young or older mice was radically different, in fact represented the two opposite extremes, when different routes of inoculation and different strains were employed.

The changes in the epidemiologic pattern over a period of four years were brought together in another publication by Traub (42). The disease had become milder but the most profound change was the change in the mode of infection and the consequences of infection at different stages of the development of the host. Intrauterine infections became the rule (previously some were infected by contact). "This shift has occurred gradually since 1935 when mice infected *in utero* were the only ones to become sick. Since 1937, however, the virus is quite harmless for such animals and produces symptoms only in suckling mice from the virus-free stock exposed to contact infection." This change may have been caused by a decrease in virulence of virus or a decrease in the vulnerability of the host (especially at the fetal level) or by combination of these factors or still other factors. Whatever the cause, the chain of events led to a progressive mutual adaptation with survival of the virus in the colony and solid immunity of the population of the colony. Similar observations were made by Haas (43). Whitney's findings (41) on the tendency of the "wild" virus to cause mostly inapparent infections in baby mice as opposed to fatal illness in adults are also in line with Traub's observations.

At present, the perfect mutual adaptation of LCM virus and fetal animal

[2] Whitney's data are also interesting for another reason. They indicate that the suckling mouse is an unsatisfactory animal for primary isolation of lymphocytic choriomeningitis virus—a very important contribution in this day of mounting use of the suckling mouse for diagnostic purposes.

appears to be a specific phenomenon. Experimental infections of embryos (cited in next section) as well as natural infections of the human fetus by several viruses attest to the high vulnerability of the prenatal animal when brought into contact with certain viruses.

Infection of the fetus under natural conditions.—It has now been well established that the virus of rubella can cause congenital abnormalities in children of mothers who have contracted this disease during pregnancy, especially in the first trimester. These include bilateral cataracts, heart disease, and dental deformities. Following the original observations of Gregg (44), similar observations have been recorded in many parts of the world (45 to 48). It should be emphasized that the extensive damage in the fetus is caused by a virus which, in the child or adult, elicits a minor illness. Congenital abnormalities have also been reported in several instances where mothers had other intercurrent virus infections during pregnancy (46, 49, 50). With poliomyelitis, measles, and smallpox there appeared to be an increased incidence of fetal death (46, 51).

It is interesting that abnormalities could be produced in chick embryos with influenza virus and to a lesser extent with the mumps virus if inoculation was done at the age of two days (equivalent to approximately first month of human gestation) [Hamburger & Habel (52)].

The foregoing illustrates how the period of life at which an animal becomes infected influences several aspects of a disease acquired by natural modes. This influence is neither static nor constant. It actually represents the net result of interaction of many variable factors and becomes manifest in a variety of ways, i.e., in difference in severity of disease, in alteration of clinical picture, in duration of the carrier state, and in the development of overt or latent infection.

EXPERIMENTAL INFECTIONS

Experimental infections differ in several respects from those occurring under natural conditions. The environments are different. The portal of entry of virus is often different. Moreover, in the latter, virus usually enters the body without the trauma which accompanies experimental induction of disease. But perhaps most important is the fact that natural infections may be influenced by the blending of natural resistance with specific immunity either passively or actively acquired. In experimental infections, it is possible to separate such effects. For this reason, infections induced in the laboratory lend themselves better to critical analysis.

Infections of the Embryo

The guinea pig.—The fetus of the guinea pig has been found to be quite vulnerable to the action of several viruses. Thus, Markham & Hudson (53) showed that amounts of submaxillary gland virus entirely innocuous for young guinea pigs could bring about death of the fetus. This disease of guinea pigs belongs to the "silent" or latent variety of infections. The

authors noted that whereas in the young animal cerebral inoculation was followed by a considerable exudate of lymphocytes and the confinement of lesions to the meninges, in the half-term fetus there was almost no cellular exudate in the meninges and virus spread to other tissues. Thus, infection of the embryo was associated with widespread dissemination.

Stritar & Hudson (54) found that the embryo guinea pig was markedly susceptible to neuro-vaccinia. Woolpert *et al.* (55) observed young guinea pig fetuses to be quite susceptible to influenza type A virus injected intracerebrally. A subsequent paper from the Ohio State University group [Dettwiler *et al.* (56)] reported that even the older fetus was more susceptible than the young guinea pig to influenza type A virus (although perhaps less susceptible than the younger fetus). The inoculations were done by the cerebral route. Following this procedure, the virus disseminated and multiplied chiefly in the lungs. The virus disappeared by the tenth day and this seemed to be coincident with the presence of antibodies resulting apparently from active immunization[3]. Newborn guinea pigs inoculated intracerebrally with this virus responded with the development of antibodies but little or no virus could be found in the brain, lung, liver, kidney, or blood. Virus multiplication in the lungs followed nasal instillation, however. Thus with birth, the guinea pig suddenly became refractory to infection by cerebral inoculation but remained moderately susceptible to infection by the nasal route. This sudden change in susceptibility will be referred to again later.

When guinea pigs were infected *in utero* and permitted to come into the world by natural birth or by Caesarean section, when desirable or expedient, virus was not demonstrated if the total incubation (pre- and post-natal) was less than 18 hours or more than six days. A minimum incubation of 12 hours *in utero* appeared to be a prerequisite for growth of virus. A period of 36 hours *in utero* seemed necessary to insure good growth of virus in the lungs. Spread of virus to other tissues tended to occur when the intrauterine period was prolonged to 48 hours. (A point which should be kept in mind is the fact that the newborn guinea pig is physiologically more advanced than are the newborn of other laboratory animals.)

This high susceptibility of the guinea pig fetus is not, however, an absolute property. According to Woolpert (57) the fetus was just as resistant to the poliomyelitis virus as was the adult animal.

The chick embryo.—The chick embryo, which has become the most useful laboratory animal for the study of viruses, offers an opportunity for further analysis of the "maturation" factor. Most of the viruses studied in the laboratory can be grown with relative ease in the embryo [Beveridge & Burnet (58)]. Yet, the majority of these seem to find the chick or chicken refractory. It is remarkable how the mere process of hatching often converts

[3] The antibody level was higher in the fetal blood than in the maternal blood. It is of interest that newborn human infants may have antibody titers to vaccinia virus which are higher than their mothers's (240).

a very susceptible host to a resistant one. Evolutionary or ecologic factors may operate in some instances. For example, the Newcastle disease virus (NDV) can infect both embryos and chickens while the mumps virus, which possesses many properties in common with NDV, infects embryos but, to our knowledge, does not infect chickens. We have observed (59) that influenza virus, which belongs to this group, was able to penetrate the respiratory mucous membranes of chicks or chickens, although it caused no signs of illness in them. It is conceivable that these viruses may have descended from a common stem virus capable of infecting chickens. As will be cited elsewhere in this review, mutations in viruses may manifest themselves in an altered pathogenicity for animals of different ages.

Changes in the Susceptibility of Chick Embryos in the Course of Their Development as Determined by Different Procedures

Data on the susceptibility of embryos of different ages to virus infections have been derived from three types of experiments: (*a*) Observation of the severity of the infection; (*b*) determination of the dosage required to induce infection; (*c*) measurement of the extent of virus multiplication.

The severity of infection.—Using the first criterion, Bang (60) found significant differences in susceptibility to infection with the pseudorabies virus in embryos inoculated on the chorioallantoic membrane at different stages of development. In the 12-day embryos whose membranes tended to develop ulcers and confluent pocks, the central nervous system was extensively involved and hemorrhage was a prominent finding. In 15-day embryos (the membranes of which had only a few scattered pocks), many nerve cells were destroyed but there was little hemorrhage. In hatched chicks, there was no hemorrhage and destruction of nerve tissue was only slight. The infection was accompanied by perivascular infiltration and neuronophagia. In the embryo, leukocytes did not usually penetrate the central nervous system. [This is somewhat analogous to the observations of Markham & Hudson (53) in guinea pigs.] Other viruses have also been found to induce more severe infections in young embryos as demonstrated by high mortality and decreased hatchability [Bang (61), Fox & Laemmert (62), Sigurdsson (63), Koprowski & Cox (64)]. High susceptibility does not always manifest itself, however, in the form of increased mortality or diminished hatchability [Bernkopf & Kligler (65), Schlesinger (66)].

Dosage required to induce infection.—The limiting dilution of virus required to infect (ID_{50}) or to cause death (LD_{50}) of embryos may vary when embryos of different ages are used [Bang (61), Crawley (67)]. The alteration in susceptibility during the transition from embryo to chick is of special importance. This change may be sudden as with influenza (68) and yellow fever (62) or gradual and projected into postnatal life as with the equine encephalomyelitis viruses (61). In fact, Bang found that the chick inoculated subcutaneously was more susceptible than was the chick embryo injected on the chorioallantoic membrane. As the chicks grew, they became progres-

sively more resistant. He thought that the explanation for this apparent discrepancy was increased availability of cells with subcutaneous route of inoculation. Still other factors were probably also operative. For, whereas the difference between the LD_{50} of the two viruses in the embryo was not too great (about 1 log), in chicks it was quite striking [about 4 logs: the LD_{50} for Eastern equine encephalomyelitis (EEE) was $10^{-9.5}$, $10^{-8.5}$, 10^{-7} at the ages of 2, 6, and 15 days, whereas the LD_{50} for Western equine encephalomyelitis (WEE) was about 10^{-5} at 6 days and about 10^{-2} at 15 days]. Thus, the response of the hatched chick differed in relation to the somewhat similar viruses, illustrating the important role played by genetic or physiologic properties of the virus in this host-virus interaction. Recent observations by Chamberlain & Kissling (69) on the occurrence of a distinct break in susceptibility of chicks to a fresh strain of EEE at two to three days suggest that differences may not only be encountered with different viruses, but with different strains of the same virus.

The influence of the "age factor" seems to be conditioned also by the route of inoculation of the embryo. Crawley (67) reported that while there was an increase in resistance of embryos to the EEE virus with increasing age above eight days when inoculated by the intraallantoic route, there was an increase in susceptibility with increasing age up to 15 days, at least, when inoculation was done into the yolk sac.

Multiplication of virus.—Other examples of the variability of the influence of the embryo's age on susceptibility to virus infection are afforded by experiments where the level of virus multiplication was the criterion. Bernkopf & Kligler (65) found that rabies virus (Pasteur strain) could grow better in embryos five or six days of age than in older embryos following inoculation of the chorioallantoic membrane. Yet, more recently, Koprowski & Cox (64) obtained no growth of another strain of rabies (Flury) in five-day-old embryos following yolk sac inoculation. This virus grew abundantly, however, in seven-day-old embryos.

The yellow fever (62) and dengue viruses (66) were found to multiply more prolifically in younger than in older embryos. Yet, better yields of influenza A virus, as measured by the concentration of hemagglutinins, were obtained in 10- or 11-day-old embryos than in 7-, 8- or 12-day-old embryos inoculated into the allantoic cavity [Miller (70)]. In contrast are the studies of vonMagnus (71) who found no significant differences between old and young embryos inoculated with the influenza A virus. The difference in the results may reflect changes in the Princeton strain of PR8 virus which was used by both authors. VonMagnus states that this virus underwent many mouse and embryo passages in his laboratory prior to this study. [It should be pointed out that for the purpose of isolation of influenza A viruses, it is recommended (58, 72) that 12- or 13-day-old embryos be used and inoculated by the amniotic route; younger embryos appear to be more sensitive to newly isolated influenza B virus (73).]

Somewhat better virus titers developed in nine-day than in seven-day-old

embryos injected by the yolk sac route with Japanese B encephalitis virus as observed by Koprowski & Cox (74).

Thus the age at which most prolific multiplication of virus was obtained varied with different viruses. An age that was optimal for one virus was less satisfactory for another.[4]

In summary, no clear-cut statement can be made about the change in susceptibility of chick embryos to virus infection in the course of their development. Analysis of the data shows that different results are obtained when one uses different criteria of evaluation, or different routes of inoculation, or different viruses. Although in general the embryo appears to be more susceptible than the postnatal animal, this is by no means a constant characteristic, as evidenced by the responses of chick embryos and newly hatched chicks to the EEE and WEE viruses (61).

Infections of the Postnatal Experimental Animal

In 1927 Andervont (75) observed that chicks were more susceptible than chickens to neurovaccinia virus administered by the corneal and intracutaneous routes. Two years later he (76) pointed out that young mice (12 to 14 days of age) were more easily infected than older mice by the virus of herpes simplex injected peripherally. Theiler (77) found that intraperitoneal inoculation of baby mice with the yellow fever virus led to encephalitis in these animals whereas similar inoculation of adult mice was ineffective. On the basis of these and other observations (78), it was assumed, pretty generally, that the young animal was more susceptible than the mature animal to virus inoculations. A precise definition of the nature and significance of "the age factor" in resistance was lacking, however, until the appearance of the classical report of Olitsky, Sabin & Cox (79) and the comprehensive experiments of Sabin & Olitsky (80 to 85) which followed.

Differences encountered with different routes of inoculation in mice.— These investigators (79) noted that while 14-day and 1-year-old mice were almost equally susceptible to the virus of vesicular stomatitis injected intracerebrally, the young mice were much more susceptible than the older ones when injected intranasally, subcutaneously, or intramuscularly. Both groups appeared to be refractory to virus introduced intraperitoneally. The remarkable resistance of the older mice was not associated with either nonspecific or specific antiviral substances in the serum.

With EEE virus they again found no appreciable differences when the cerebral route was employed and only slight differences when virus was instilled intranasally. The WEE virus was essentially equal in pathogenicity for 21-day and one-year-old mice following cerebral injection, but 15-day-old mice appeared to be somewhat more susceptible as demonstrated by

[4] The selection of embryos of a certain age for large scale operations, such as preparation of vaccine, is based on several considerations including yield of virus per unit amount of fluid or tissue, total amount of fluid or tissue, ease of handling, and nonspecific mortality.

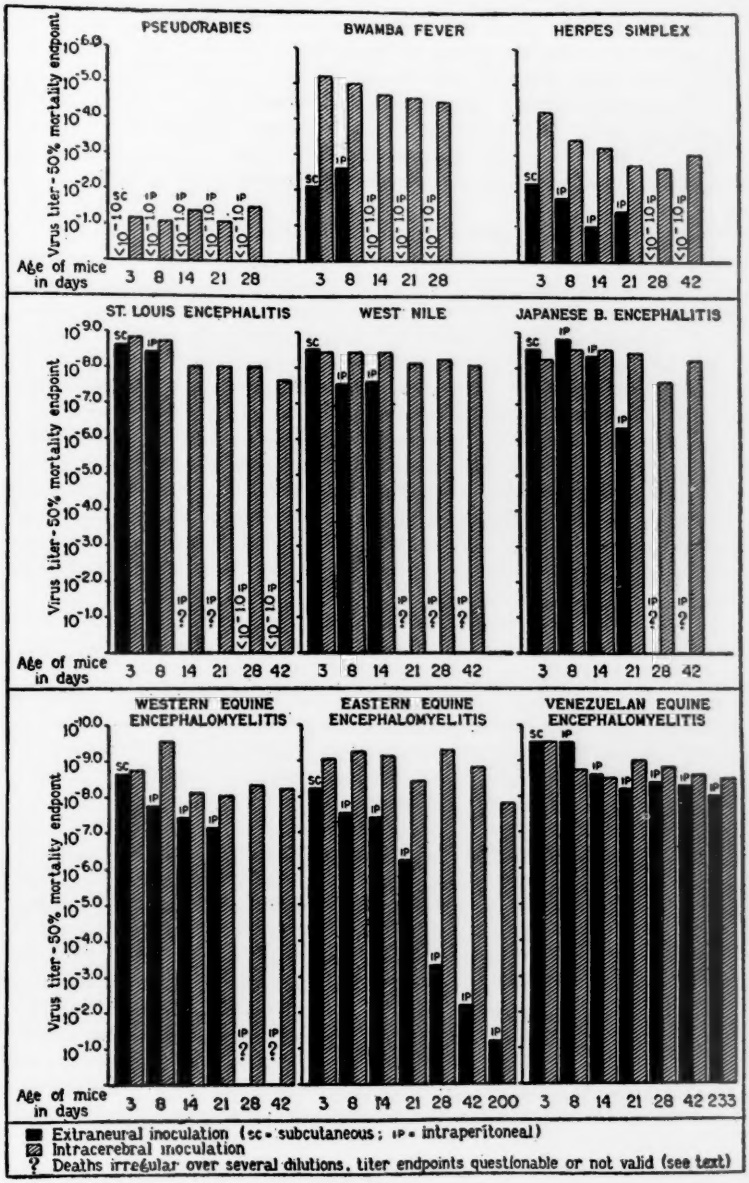
PSEUDORABIES
BWAMBA FEVER
HERPES SIMPLEX
ST. LOUIS ENCEPHALITIS
WEST NILE
JAPANESE B. ENCEPHALITIS
WESTERN EQUINE ENCEPHALOMYELITIS
EASTERN EQUINE ENCEPHALOMYELITIS
VENEZUELAN EQUINE ENCEPHALOMYELITIS
Virus titer - 50% mortality endpoint
Age of mice in days
Extraneural inoculation (sc = subcutaneous; ip = intraperitoneal)
Intracerebral inoculation
? Deaths irregular over several dilutions, titer endpoints questionable or not valid (see text)

FIG 1. Comparison of susceptibility of mice of various ages to infection with different neurotropic viruses.

shorter incubation periods. After inoculation by the nasal route, there was a more pronounced difference in susceptibility of 15- or 21-day-old and the mature animals (but not so remarkable as with the vesicular stomatitis virus).

Using the St. Louis virus, O'Leary, Smith & Reames (86) noted that a significant change in susceptibility of mice occurred between two and three weeks of life when measured by the intraperitoneal mode of inoculation. There were few, if any, age differences demonstrable with cerebral or nasal inoculations.

Differences obtained with different neurotropic viruses and with "fresh" and passaged strains of the same virus.—Figure 1 (see page 257) taken from the paper of Lennette & Koprowski (87) illustrates very clearly the differences in susceptibility of mice of different ages to peripheral inoculations with several neurotropic viruses. The data pertaining to the pseudorabies virus are of doubtful significance because of the very low titer of the virus. King (88) had found previously that a more potent strain could elicit illness even in adult mice following extraneural injection. A comparison of the results obtained using the Bwamba or St. Louis encephalitis viruses with those obtained with the Venezuelan encephalomyelitis virus brings out the magnitude of the variation in the effect of age in relation to different viruses. The results expressed by question marks pertain to irregular mortality ratios. Some of these irregularities obtained in older mice with lower dilutions of virus may represent the effect of the interference phenomenon. This factor will be discussed in the third part of the review.

Melnick *et al.* (89) confirmed recently Lennette & Koprowski's findings with the West Nile virus. The observations on the decrease in susceptibility to peripheral inoculation of herpes simplex virus were confirmed by Kilbourne & Horsfall (90). These investigators noted, however, that a marked decrease in susceptibility also occurred in the brain tissue itself when a freshly isolated strain was employed for the determination. At 21 days of age, mice were equally resistant to intraabodminal and intracerebral inoculation of such a virus. Reference has already been made to the greater resistance of older mice to lymphocytic choriomeningitis virus freshly isolated from a mouse colony as compared with a guinea pig-passaged strain. In contrast, data obtained from studies on the rabies and EEE viruses indicate that age-induced resistance became more apparent against passaged strains than against newly isolated viruses.

Casals (91) showed that while 20-day-old mice were more susceptible to peripheral inoculation of fixed rabies virus than were 60-day-old animals, the two age groups were equally susceptible to the street virus injected peripherally. This author noted that seven- to nine-day-old mice were more susceptible than older mice to the fixed or street strains of rabies virus as determined by cerebral or extraneural modes. The work of King (92) revealed that although, in general, adults were more resistant than infants to the EEE virus, this resistance was the more marked when the mice were challenged with fixed rather than fresh strains. The subcutaneous mode of

inoculation was at least as effective as the intranasal or intraocular when the unadapted virus was used but proved to be almost completely innocuous with the fixed virus. Thus, there is no consistency in the results obtained with adapted and unadapted strains and the differences may well reflect certain innate properties of virus strains irrespective of the degree of adaptation. This possibility is supported by the observations of Kissling (93) that the susceptibility of weaned mice to peripheral inoculation varied markedly for several strains of freshly isolated EEE virus.

Regarding the behavior of strains with different tropism, one may cite the observations of Bugher (94) on the yellow fever virus. He found marked differences between neurotropic and viscerotropic strains with respect to the incubation periods and survival times on cerebral inoculation of mice of all ages. In contrast, mice under nine days of age exhibited equal susceptibility to subcutaneous inoculation of these strains, the survival periods approximating those of mice injected intracerebrally with the viscerotropic virus. Thus, neurotropism of the yellow fever virus does not seem to represent an enhanced capacity for neuroinvasiveness in the baby mouse.

In summary, the appearance of resistance in mice to inoculation of neurotropic viruses is a variable occurrence. It may be limited to peripheral pathways or it may involve the central nervous system (CNS). Differences obtained with different viruses, with adapted and "fresh" viruses and with different strains of the same virus, indicate that this occurrence is dependent on properties inherent in the virus.

The behavior of the neurotropic viruses in animals other than the mouse.— In a paper describing a study of the susceptibility of several domestic and wild animals to WEE virus, Howitt (95) stated that young chicks (up to age of five weeks) and young turkeys were highly susceptible; older animals exhibited an irregular response. According to Watson & Smadel, (96), old hamsters were less susceptible than young adults or adolescents when injected by peripheral routes with WEE, EEE, and West Nile encephalitis viruses. Meyer (97, 98) found that young rabbits were susceptible while old rabbits were quite resistant to the WEE virus. Similar observations were made by Schlesinger, Morgan & Olitsky (99), who showed that adult rabbits inoculated subcutaneously with this virus developed a silent infection of the CNS (associated with fever) while young rabbits similarly injected died of encephalitis.

Age-induced resistance may often manifest itself by the development of silent or subclinical infections rather than by absolute refractoriness. An EEE virus isolated from a pheasant caused paralysis and death in adult pheasants after intramuscular inoculation. This virus was very pathogenic for young chicks injected subcutaneously but could induce only subclinical infections in young hens [Sellards, Tyzzer & Bennett (100)]. Duffy & Sabin (101) called attention to the fact that although rats at the age of 21 days remained well following inoculation of the St. Louis virus by the cerebral or nasal routes, the virus multiplied in their brains. Seven-day-old

animals injected by the same routes succumbed to encephalitis. More recently, Duffy (102) extended these observations to the Japanese B encephalitis virus. Following cerebral inoculations, 7-day-old rats were uniformly vulnerable, while some 12-day and all of the 21-day-old rats failed to show signs of illness. Intraperitoneal inoculation revealed that the 7-day-old rats were highly susceptible (100 per cent mortality) and the 12-day-old ones were highly resistant.

Thus a given virus could elicit illness in the young and mature animals of one species (EEE virus in the pheasant and St. Louis and Japanese B viruses in the mouse on cerebral injection) but only in the very young animals of another species (EEE in the chicken and St. Louis and Japanese B in the rat).

Determination of age-induced resistance to Theiler's virus in mice from infected and virus-free colonies.—Reference has already been made to Theiler's encephalomyelitis virus in connection with latent infection. According to Olitsky (103, 104), the virus was not detectable in the intestines of "normal" baby mice up to the age of 12 days; was found irregularly in 20- to 25-day-old mice; was present with great regularity in "normal" adults; and was again demonstrable irregularly in old mice (six months or older). The absence of virus in the young mice was not a result of natural resistance of these animals, for as observed by Theiler & Gard (105, 106), younger animals appeared to be more susceptible than older ones to inoculation of this virus. Evidence of passive immunity transmitted through the placenta and by milk has been obtained by vonMagnus (107) and this finding, combined with her observation that mice were able to utilize the immunizing capacity of milk only during the first 12 days of postnatal life, suggests that the apparent absence of virus may be due to prevention of infection or masking of virus by the antibody.

Evaluation of the susceptibility of mice to Theiler's virus has been hindered by the presence of latent infections in many mouse colonies. The recent successful establishment of colonies free from this virus (108, 109) made such an evaluation possible. According to vonMagnus (110), mice from the virus-free colony were extremely susceptible to inoculation by the intracerebral, intraperitoneal, subcutaneous, oral, and intramuscular routes at one day to one week of life. At two to three weeks they were still highly susceptible to cerebral and intraperitoneal inoculations, but considerably less susceptible (20 per cent) to the feeding of virus and quite resistant to subcutaneous and intramuscular injection. Older animals succumbed only to cerebral injection. Ordinary mice (from colonies with latent infection), were moderately susceptible (59 per cent) to feeding and slightly susceptible to subcutaneous (19 per cent) and intramuscular (18 per cent) inoculation of the virus at one day to one week of age. Thereafter, they proved to be quite resistant to infection by all peripheral routes. On cerebral inoculation, mice up to four weeks of age were as susceptible as those from the virus-free colony. Older animals appeared to be more resistant.

The findings of this study are of special significance because they represent an exploration of the development of natural resistance with age in the presence and absence of specific immunity. They offer a clue to the problem of at least one latent disease.

Alteration of the Clinical Picture as a Manifestation of Age-Induced Resistance

The Western equine encephalomyelitis virus.—Sabin & Olitsky (84) noted a strikingly different clinical picture in mice aged 15 and 21 days when inoculated intramuscularly with the WEE virus. While 80 to 90 per cent of the former succumbed with encephalitis and only 10 to 20 per cent developed flaccid paralysis, 70 to 80 per cent of the latter developed flaccid paralysis. At the age of one month, 90 per cent of the mice resisted such intramuscular injection (but direct injection into the sciatic nerve resulted in paralytic disease). It is conceivable that more of the 15-day-old animals would have shown paralysis had not encephalitis and death intervened. Comparison of the periods at which encephalitis, death, or paralysis occurred after inoculation (111) suggests that such a conjecture is probably correct.

The Coxsackie viruses.—Several viruses are definable by little more than their fastidiousness as regards host age. The Coxsackie viruses (112, 113, 114) are said to infect only baby mice and baby hamsters by several routes, including the oral (115). Data obtained by Goldblum (116), while indicating a general trend toward decreased susceptibility to several Coxsackie viruses during the first 14 days of postnatal life, reveal some differences in the rate of loss of susceptibility to different Coxsackie viruses. For example, the ID_{50} measured by the cerebral route was 10^{-8}, $10^{-7.4}$, and $10^{-6.5}$ at the first, fifth, and eighth days of life for the Texas virus, while the corresponding values for the Easton virus were $10^{-5.5}$, $10^{-3.6}$, and $10^{-1.6}$, and those for the Ohio virus were $10^{-5.4}$, 10^{-2}, and less than 10^{-1}.

Pappenheimer *et al.* (117) presented a very interesting observation which shows that adult mice can become sick after inoculation with at least one Coxsackie virus (Connecticut 5 virus). Their data explain the occasional deaths in adult mice observed in this laboratory and by others following injection of this virus for the purpose of immunization. As shown by these investigators, following intracerebral, intravenous, intramuscular, subcutaneous, and intraperitoneal inoculation, adult mice developed only pancreatitis with a transient hepatitis instead of the usual encephalitis, myositis, and generalized infection including pancreatitis seen in suckling mice.

Tumor-producing viruses.—The extensive studies of Duran-Reynals have revealed that inoculation of tumor viruses leads to entirely different clinical pictures in young and old animals. This investigator (118) demonstrated that following intravenous injections of Rous sarcoma virus, chicks developed principally blebs and hemorrhages, largely in viscera, and succumbed rather quickly. Pullets responded with both tumor nodules and

hemorrhagic lesions. Adult chickens were rather resistant and many formed no lesions, but when lesions developed they were mostly neoplastic. Neoplasia was thus an expression of the resistance acquired with age. Rous sarcoma virus could infect ducks provided a large amount was injected within 24 hours of hatching (119). The development of resistance in ducks was so sudden that it could be detected in the space of hours rather than days. The refractory state of ducks to the original chicken virus was shown to be related to the natural resistance of the species and not to humoral substances (120). One of the duck-adapted viruses (121) brought about vascular lesions in the CNS of young ducklings (never in older birds)—a feature associated with certain destructive "neurotropic" viruses rather than tumor-producing viruses. This duck variant (obtained from Rous sarcoma virus of chickens which did not induce lesions in the central nervous system vessels) was practically innocuous for chickens but could cause illness in young chicks and young and adult ducks. Alteration of the clinical picture was also reported in chicken lymphomatosis. Blakemore (122) observed that a highly virulent strain of lymphomatosis induced acute necrotizing and inflammatory lesions in chicks and a delayed neoplasia in the older birds.

In analyzing the many factors which determined the different types of illness in rabbits infected with the Shope papilloma virus, Duran-Reynals (123, 124) found that age played a very important role. In general, the baby rabbit tended to succumb with acute inflammatory and proliferative lesions whereas the adult responded with the development of a relatively mild fibroma.

Age-induced resistance of some animals to certain viruses, then, may manifest itself in the form of an illness which differs clinically and pathologically from that elicited in the younger and more susceptible animal.

Other Manifestations of Resistance Acquired with Age

Age-induced resistance may manifest itself in the form of absent infection, inapparent infection, subclinical infection, or a changed clinical picture. Other types of manifestation have emerged from research with the tumor-producing viruses.

The effect of age on the transmissibility of mammary cancer was evident in the experiments of Bittner (125). A higher incidence occurred in the daughters of mothers who developed the tumor early in life. Gross (126) noted that inoculation of the mouse leukemia agent into mice less than 12 hours old led to the development of "spontaneous" leukemia in half of them at $8\frac{1}{2}$ to 11 months. In contrast, inoculation of mice three to six days old resulted in a considerable delay of the appearance of leukemia; none of the animals developed leukemia before the age of 18 months.

Observations of Duran-Reynals (127) suggest that resistance of the host may influence mutation in the Rous sarcoma virus. Variants were

obtained more easily if the virus was grown in mature chickens rather than young chicks.

The Variability of the Direction of Change in Susceptibility with Growth and Development

In the foregoing sections dealing with experiments in the post-natal animal it was shown that as certain animals grew or matured they became more resistant to some viruses, and that the break in susceptibility was either sudden or gradual and progressive. This inverse relation between susceptibility and host age is not limited to the viruses cited. A similar situation was noted with the influenza virus (128) and the rabbit-adapted hog cholera virus (129). This pattern is not universal, however, for there are several instances in which the direction of change in susceptibility appears to be reversed or in which there are intermittent chronologic zones of increased or decreased resistance.

The pronounced resistance of infant mice to fresh strains of the lymphocytic choriomeningitis virus has already been referred to (41). In connection with the changed clinical picture of mice infected with the Connecticut type of Coxsackie virus (117), it is of interest that over the age range of approximately 5 to 21 days, mice appeared to be relatively resistant. They became susceptible again (i.e., developed pancreatitis) at the end of the suckling period and remained susceptible until the age of eight to nine weeks. This recalls to mind the observations with rabbit pox (33).

Fenner (130) found that the mortality from ectromelia was higher in baby mice and old mice (about a year old) than in eight-week-old mice.

Additional reasons against generalization in describing the change in susceptibility with age are provided by the observations on the behavior of the poliomyelitis virus. Although, as reported by Pinto (131), young cotton rats (6 to 10 days of age) were more susceptible to peripheral inoculation of the Lansing strain of virus than were adults, infant mice were more resistant than adults even on cerebral injection [Sabin (132), Dalldorf (133)]. It should be mentioned that in general the cotton rat is more susceptible and vulnerable to the Lansing strain of poliomyelitis than is the mouse. It may be more than a coincidence that poliomyelitis virus (with its tendency to cause inapparent disease) and the "wild" lymphocytic choriomeningitis virus (with its latency in mice) should have the common feature of low pathogenicity for the young animal.

That variants highly pathogenic for baby mice can be obtained was shown by Casals, Olitsky & Anslow (134, 135), who adapted the MEF_1 strain (a Lansing-type poliomyelitis virus) to baby mice by means of rapid serial passages. The adapted virus was highly infective for infant mice. The baby mouse, once faced with the mutant of the virus which originally was of low pathogenicity for it by the cerebral route, could be rendered sick not only by the cerebral route but even by peripheral routes, as was revealed

by the experiments of Hammon, Cheever & Sather (136). But here again it was not a matter of the youngest being the most susceptible. Mice one to three days of age were fairly resistant to intraperitoneal inoculation, whereas mice between the ages of 4 and 15 days were quite susceptible to this mode of injection with the virus adapted by Casals *et al.* (134).

Some Practical Considerations

Reference should be made to the practical application of the "age factor." The peripheral route of inoculation in infant or young mice has been utilized to great advantage in neutralization and protection tests (136 to 142). The peripheral neutralization test appears to be superior to the usually employed cerebral procedure. It is more sensitive and capable of detecting smaller amounts of antibody than the more severe cerebral test.

The CNS of baby mice was found to be an excellent source of antigen for complement fixation tests with the MEF_1 strain (135). Finally newborn mice have become a very useful host for the isolation of some viruses (90, 143) and the isolation and identification of the Coxsackie viruses (112, 113, 144, 145, 146).

CONSIDERATION OF THE POSSIBLE MECHANISMS IN THE INFLUENCE OF AGE ON SUSCEPTIBILITY TO VIRUS INFECTION

Various aspects of natural resistance against infectious diseases are extensively discussed in the books of Perla & Marmoston (147) and Burnet (148). As regards the "age factor," there are, however, but a few reports in which an attempt was made to correlate age-induced differences in susceptibility to virus infection with fundamental or special mechanisms. One often sees such terms as "serologic maturation" and "physiologic adjustment" but the meaning of these expressions, especially the second, is obscure.

Consequent to the analytical studies of Sabin & Olitsky (80 to 85) some 15 years ago and with the accumulation of specific observations by many investigators, it has been possible to recognize and sometimes define separate factors and principles. The inescapable fact which has emerged from the studies is that resistance acquired with growth and maturation is not an absolute phenomenon but a specific development dependent on time, the species characteristics of the host, mode of infection, inherent properties of the virus, and mode of dissemination of the virus within the host. These aspects are discussed in the second section of Part III. The first section of this part concerns certain mechanisms and factors which are believed to affect virus growth or host-virus interaction.

Fundamental and Special Mechanisms

Metabolism.—There is available a large body of information on the changes in metabolic patterns and in the energy-controlling systems during

the process of growth and specialization (149 to 175). It is difficult, however, to correlate the reported changes in susceptibility with these biochemical alterations for several reasons.

(*a*) The knowledge of metabolic and energy requirements of animal viruses is very meager. Most of the information pertains to the effect of temperature on virus growth (58, 63, 68, 70, 73) (in embryos better virus multiplication has been obtained at 35° to 37°C. than at 39°C.)[5] and the behavior of several viruses in complex tissue culture (176, 177, 241, 242, 243). In connection with the former, Enders & Pearson (68) suggested that the high body temperature of the hatched chick may be a factor in resistance of the chick to influenza virus. They found that the virus multiplied in tissue culture of two-day-old chick lung at 37°C. but not at 41°C. Regarding the tissue culture experiments, the work of Zinsser & Schoenbach with WEE virus suggested that virus multiplication required actively metabolizing cells (241). From the experiments of Plotz (242, 243) with fowl plague and vaccinia viruses, and of Fox (176) with the yellow fever virus, it appeared that active proliferation of cells in culture was a requirement for good virus multiplication. Yet, according to Zinsser & Schoenbach (241), maximum virus titers in the tissue culture fluid were not reached until about 12 to 24 hours after cessation of tissue respiration. Furthermore, Pearson (177) reported that active tissue proliferation was not a prerequisite for the multiplication of Theiler's GDVII virus. He obtained good growth in tissue cultures of postnatal mouse brains. It should be noted, however, that only brains secured from mice under 10 days of age could support multiplication of this virus. The limiting factor in this system is unknown. One may suggest such possible explanations as the greater potential ability of young brain cells to undergo multiplication [mitotic activity in brains of young animals has been observed (185)], or the alteration of the physiology of the brain with growth and maturation (some of these changes will be mentioned under "Inhibitory Substances"; see also references (151 to 154, 174)). Whatever the nature of the limiting factor, it is important to keep in mind that while it plays a role in tissue culture it appears to be ineffective in the intact animal. GDVII virus is capable of multiplication in the brains of intact adult mice.

Some light on the problem of the relation of energy-transforming systems to virus multiplication has been shed by the work of Howe and associates (for summary see 178) on regenerating nerve cells. It was found that refractoriness of this cell to the poliomyelitis virus appeared to be associated with the mobilization of certain enzyme systems for the production of Nissl substance.

There is more information available on the metabolic requirements of rickettsiae and it may therefore be less hazardous to venture some cor-

[5] But even this is not a constant feature, for as shown by Schlesinger (66), there was little difference in the growth of the Hawaiian strain of dengue virus in embryos incubated at 35°C. and 39°C.

relative speculations. It is conceivable that the higher rate of oxygen uptake by vascular tissue of young rats compared with older animals (155) and the greater riboflavin content of young rats (riboflavin content of muscle was higher at 30 days than at 60 days of life) (156) may be at least a partial explanation for the greater resistance of young animals to typhus rickettsiae which have been found to prefer tissues in which the metabolic rates are low (179).

(*b*) Determinations of both chemical and autarcelogic changes have been carried out usually on the whole animal or a tissue rather than on the individual cells concerned in the ultimate phase of infection. This should especially be kept in mind when one feels tempted to project observations made in studies with phages to other host-virus systems associated with specialized organs, hormones, etc.

(*c*) The criterion of resistance has varied in the numerous reports and with few exceptions little is known about the level of virus multiplication. It must be reemphasized that the changes occurring in the metabolism of cells may have entirely different effects on such separate phenomena as virus growth, dissemination, damage to cell, survival and recovery of cell, etc.

(*d*) Susceptibility or resistance may be the sum total of antagonistic and complementary influences of several systems exhibiting different metabolic patterns, the knowledge of which is anything but adequate.

Recent work with radioactive isotopes suggests that certain viruses can stimulate carbon and phosphorus uptake by tissue (180 to 183). It is, therefore, not unlikely that the potential metabolic activity of a tissue may be of equal, if not greater, importance than the actual activity prior to infection with a virus. At this point, we are tempted to ask a speculative question: It is assumed that multiplication of virus is predicated on its being able to divert enzyme activity from the normal function to the synthesis of elementary bodies; is it not possible that enzymes of less differentiated or specialized tissues can be more easily diverted?

It is of special interest that incorporation of C^{14} into two amino acids (lysine and histidine) was inhibited by a virus (183), and that these two amino acids were previously shown to inhibit growth of this virus (182). It may also be significant that the concentration of histidine in the brain appears to increase with maturation (184).

There is obviously great need for more extensive and correlative studies on the chemical and physiological aspects of virus multiplication. Better understanding of these problems may be expected from the use of single type cell tissue culture.

Anatomical and cytological changes.—(See 149, 185, 189 to 195).

Localization of virus in skin.—Foley (196) noted differences in the rate or intensity of local reactions caused by several viruses in the skin of animals of different ages. According to Pearce *et al.* (197) vaccinia virus produced typical local reactions in adult rabbits but in young animals caused general-

ized infection usually in the absence of local lesions. Similar findings were obtained by Ørskov & Anderson (198). These findings do not reflect resistance on part of the young skin but rather a state of low reactivity and an inadequate capacity for localizing certain noxious agents and are in accord with other bacteriological findings (147, 199).

The hemoencephalitic barrier.—It has been reported (200) that inoculation of various innocuous substances into the brains of adult mice while virus was circulating in the blood (in animals ordinarily remaining free of encephalitis consequent to peripheral injection of virus) brought about invasion of the brain and encephalitis. The procedure was thought to damage the blood-brain barrier, causing adult mice to lose their physiologically acquired resistance. Some interesting considerations of the problem of this barrier in virus diseases were given by Hurst (201), who presented findings on the dissemination of two neurotropic viruses. This author called attention to the importance of consideration of such factors as the ability of animals of different ages to support multiplication of the virus outside the CNS, the amount of virus reaching the CNS after peripheral inoculation, and the relative susceptibility of the CNS to small amounts of virus. He also noted that while one of the viruses (EEE) could not invade the CNS of the resistant older animals [this is in accord with the finding of Sabin & Olitsky (85)] the other (louping ill) could do so and cause an inapparent infection of the brain. Thus age-induced resistance to neuroinvasiveness of certain viruses was related to some intrinsic changes in the cerebral blood vessels. This phenomenon appears to be specific and does not apply to all neurotropic viruses. On the basis of the determination of the incidence of encephalitis under the influence of substances reputed to affect the permeability of the blood-brain barrier, Hurst concluded that the process by which the virus invades the CNS from the circulation is independent of simple permeability.

Antibodies.—An excellent review on quantitative and qualitative differences in the serologic response and in antibodies associated with maturation was published by Baumgartner (202). While such differences have merit in the realm of controlled experimental determinations, one should not ascribe to them the variable serologic patterns of animals (especially man) under natural conditions. Outside of the laboratory, these may actually reflect differences in the frequency of exposures to the virus rather than differences resulting from maturation.

Morgan (203) found that the rate of antibody formation in mice immunized with formalized EEE increased as age increased. She felt (204) that the degree of peritoneal resistance to this virus at different ages of the host may have been a reflection of the capacity of the organism to develop antibodies. Casals, on the other hand, noted (205) that the ability to form antibodies was not necessarily related to the development of resistance. He demonstrated that while 60-day-old mice were more easily immunized than 20-day-old animals with a virulent rabies vaccine, both groups showed approximately the same immune response to a nonvirulent vaccine when

challenged by the muscular route. Seven-day-old mice remained susceptible. It is of special interest that these susceptible vaccinated seven-day-old mice possessed circulating antibodies in significant concentrations (sometimes equal to that of 20- and 60-day-old mice) and yet failed to withstand challenge with an amount of virus which was innocuous for the 20- and 60-day-old animals. Thus antibodies could not compensate for the high vulnerability of the very young animals.

The experiments of Sabin & Olitsky (80) revealed that the development of regional resistance was not dependent on antibodies. Likewise, there was no reason to suspect that the breaks in susceptibility occurring at different times with several neurotropic viruses (87) were in any way related to immunologic maturation. It would be difficult to explain the appearance of peripheral resistance to one virus at eight days of life and the complete failure of the resistance to another virus to develop even in old mice on the basis of immunologic maturation. Still, the changes in the susceptibility of Rous sarcoma appeared to be associated with the occurrence of an antibody-like substance (206). The same situation seemed to exist with vaccinia infection in chickens (207).

Thus, while the precise role of serologic or immunologic maturation in the influence of age on susceptibility remains obscure, it is possible that under certain conditions this phenomenon may affect to some extent the balance of a host-virus interaction.

Hormones.—The profound changes taking place in the endocrine system with growth and maturation make it seem unlikely that the physiologic mechanisms determining resistance to virus infections would be spared their influence. Work of several investigators indicated that estrogenic substances could enhance the state of resistance (208, 209). Recently, Kalter (210) found that testosterone caused an increase in the growth of influenza virus in the lungs of mice (in a sense, it decreased resistance of these animals).

A striking change during the very early phase of postnatal life occurs in the adrenals. Shortly after birth, the adrenal cortex of several animal species, including man undergoes involution. Subsequently, it begins to develop gradually into the anatomically and physiologically typical cortex of the adult. There is no direct evidence that these changes influence susceptibility. It is important, however, that alterations in susceptibility can be obtained with cortisone. Kilbourne & Horsfall (211) reported that cortisone reduced resistance of adult mice to a Coxsackie virus and also stimulated growth of influenza and mumps viruses in embryos (212). We (213) have not been able to confirm this last observation. Schwartzman (214) demonstrated an enhancing effect of cortisone on the susceptibility of hamsters to poliomyelitis. A similar effect was obtained by Smith, Murphy & Mirick (215) in mice infected with the PVM[6] virus. Foster *et al.* (216), and Ainslie, Francis & Brown (217) found that ACTH increased the susceptibility and

[6] Pneumonia virus of mice.

vulnerability of monkeys to poliomyelitis. This predisposing effect of ACTH to more severe poliomyelitis is in keeping with the observed relationship of stress (mediated by the pituitary and adrenal glands) to increased susceptibility to paralytic poliomyelitis. Final conclusions on the influence of these hormones on susceptibility is not yet warranted. The data are both too new and too meager.

Inhibitory substances.—Parker (218) observed that fibroblasts in tissue culture grew less abundantly after treatment with adult serum as compared with infant's serum. Twenty-five years ago, Baker & Carrel (219) found the same thing using young and old chicken sera. They thought that the inhibitory effect was due to a lipid fraction—perhaps lecithin. Lipids from serum were also found to inactivate or inhibit certain neurotropic viruses (220). The nature of these lipids is unknown. Neither is it known to us whether they increase with increasing age. However, it has been established that certain lipids increase in concentration as the animal grows. Williams and associates (221) found among various changes an increase in concentration of cephalin in all rat tissues studied. Changes in serum lipids with age were reported by Shope (222). More recently, Folch (223) indicated in a personal communication that cerebrosides and proteo-lipids were absent in brains of newborn mice and first made their appearance at 7 to 10 days after birth. Subsequently, their concentration increased steadily.

Other types of virus-inhibiting substances have been reported. One of them (224), which appears to be complement or a complement-like substance, is somewhat selective in its activity. It has been reported that the concentration of complement in certain animals increases with growth and maturation (225, 226).

A polysaccharide specifically active against Theiler's virus was obtained by Mandel (109) from mouse intestines. Its concentration increased with age.

There is need for more research on the problem of antiviral substances of the body and on their possible role in natural immunity.

Certain virus factors.—Serial passages of virus have resulted in adaptation of viruses to animals of ages previously considered refractory (134, 143, 227). Schlesinger & Frankel (228) listed several features associated with the adaptation of a strain of dengue virus to adult mice. They found that the transformation was gradual and reached its climax at the same passage number in the suckling mouse series and in the adult mouse series of passages initiated in infant mice. Adults were apparently no better equipped than the infant mice to give rise selectively to particles of the modified (i.e., adult pathogenic) kind. This finding is at variance with the observations of Duran-Reynals on the Rous sarcoma virus adaptations [better adaptation to new host if virus came from older donor animal (127)], again illustrating the hazards of generalization, no matter how attractive.

Schlesinger & Frankel observed that adult mice appeared more resistant when inoculated with lower, rather than higher, dilutions of virus from the

TABLE I

LOCALIZED BARRIERS AS FACTORS IN PREVENTING OR ARRESTING INVOLVEMENT OF THE NERVOUS SYSTEM BY CERTAIN VIRUSES

Virus	Host	Route of Inoculation	Susceptibility of Young and Old	Probable Site of Barrier in Resistant Animals
Vesicular Stomatitis	Mouse	Intracerebral	Equally susceptible	None
		Intraocular	Young, encephalitis regularly Old, more than 90 per cent resistant	Retina
		Intramuscular Subcutaneous (pad)	Young, ascending myelitis Old, 100 per cent resistant	Muscle or myoneural junction Epithelium or nerve endings
		Intranasal	Young, encephalitis regularly Old, resistance varies from 50 to 90 per cent	In central nervous system, anterior olfactory region
	Guinea Pig	Intracerebral	Young guinea pigs and mice equally susceptible; old guinea pigs slightly less	None
		Intramuscular Intra- and subcutaneous (pad)	Young and old resistant	Myoneural junction and specialized nerve endings
		Intranasal	Young, occasionally encephalitis Old, 100 per centresistant	Young, (a) Between olfactory cortex and remainder of brain; and (b) between diencephalon and remainder ot brain Old, generally between nasal mucosa and olfactory bulbs
Eastern Equine Encephalitis	Mouse	Intracerebral	Equally susceptible	None
		Intranasal	Equally susceptible	None
		Intramuscular	Young, 95 per cent encephalitis, 5 per cent ascending myelitis Old, 95 per cent resistant beyond 3 months of age	Blood vessels (generally)
Western Equine Encephalitis	Mouse	Intracerebral	Equally susceptible	None
		Intranasal	Old and young susceptible Old resistant to smaller doses	?
		Intramuscular	Young: 15 days, majority encephalitis; few ascending myelitis; 21 days, 60 per cent ascending myelitis; 20 per cent encephalitis; 20 per cent resistant 30 days and older, 90 to 100 per cent resistant	Blood vessels Muscle or myoneural junction
St. Louis Encephalitis*	Mouse	Intracerebral	Equally susceptible	None
		Intranasal	Equally susceptible	None
		Intramuscular	Young, some encephalitis; others ascending myelitis Old, resistant (with few exceptions)	Not determined

* Unpublished observations made in collaboration with Dr. Robert Ward.
Reproduced from Sabin, A. B., *J. Pediatrics*, 19, 596 (1951).

early passages. This paradox disappeared once virus became adapted to the adult mouse. These results probably represent interference of unmodified with modified virus particles. A similar explanation seems in order for the irregular mortality ratios obtained with more concentrated inocula in mice exhibiting partial resistance to certain neurotropic viruses (87). The same most likely applies to the observation of Overman & Dortch (229) that high dilutions of Semiliki Forest virus were more effective in causing encephalitis in adult rabbits than were low dilutions following ocular inoculation. Thus the interference phenomenon may play an important role in the operation of age-induced resistance as well as in the determinations of this resistance. This important problem is being studied further by Schlesinger.

Regional and "Subtotal" Resistance

The experiments of Duffy & Sabin with the St. Louis encephalitis virus in rats (101) and of Kilbourne & Horsfall (90) with freshly isolated herpes simplex virus in mice revealed the development of total or general resistance of the animal with maturation. For the most part, however, all experiments with other hosts and viruses suggested that resistance was limited to tissues outside the CNS. The greatest single contribution to our understanding of the changes in susceptibility that occur with growth and maturation of the host was the demonstration by Sabin & Olitsky of local zones of resistance (80 to 85). These investigators showed that as certain animals grew they developed regional barriers to the progression of certain neurotropic viruses. The barriers appeared at different times. Their sites are shown in Table I. The development of such local blocks was apparently not predicated on the development of sexual maturity, for as tested by Sabin (230), precocious sexual maturity induced in baby mice by means of injections of gonadotropic hormone was not associated with increased resistance. The appearance of at least some of these barriers was, however, conditioned by nutritional factors. Sabin & Duffy (230, 231) found that deficiency of vitamin B complex in the diet and especially a quantitatively inadequate diet (containing the vitamins) could prevent or delay the development of the resistance to neuro-invasiveness.

These observations are reminiscent of (but not necessarily analogous to) the lowered resistance to typhus rickettsiae noted by Pinkerton & Bessey (232) with riboflavin deficiency in rats, but are contrary to the findings with certain viruses (233 to 237) where cerebral inoculations have been used. This suggests the possibility that, in part at least, different patterns of host-virus relationship under the influence of the "maturation factor" may reflect differences in the metabolic pathways pursued by different viruses. One must keep in mind, however, that these experiments may denote different mechanisms, such as invasion of the motor nerve ending as opposed to growth of virus in a motor neuron.

Regarding the question of regional defense blocks, one is tempted to engage in some speculation. Could not these changes be another manifesta-

tion of the phylogenetic and ontogenetic order of change noted in the development of enzymes and in the metabolic pattern in the nervous system? [A lucid treatment of this subject will be found in Himwich (152); for a review of the problem of myelination in the ontogenetic development, consult Langworthy (238).] The order of appearance of barriers to the spread of vesicular stomatitis virus in the mouse (peritoneal, leg muscle, the eye, the anterior olfactory region) may perhaps parallel certain ontogenetically controlled chatges. This order of change is not constant, however, as may be inferred from vonMagnus' data (110) with Theiler's virus. In her experiments, resistance to neuroinvasiveness occurred in the muscle prior to the peritoneum.

Recently it has been shown from a different experimental direction that the retinal cells of adult and infant rabbits respond differently to virus inoculation. According to Overman & Dortch (229), inoculation of Semliki Forest virus into the vitreous of baby rabbits produced focal destruction of the retina with a mild generalized inflammatory response after four days, while the same inoculation into adult rabbit eyes caused no discernible specific reaction during the period of viral multiplication, but appeared to induce an abortive mitosis in the visual cells after a prolonged interval. Whatever the mechanism of the altered response, the fact that an altered response exists is consistent with the development of a physiologically engendered local change in susceptibility. It would have been of interest to know the levels of virus multiplication reached in eyes of infant rabbits as compared with those in the eyes of adults; these data are not given.

It is often assumed that increased resistance developing with increased age is bound up with the inability of the virus to grow vigorously or well at the site of inoculation. This has been found partly true for vesicular stomatitis virus in the mouse [no multiplication at site of inoculation in muscle (81)]. The resistance to neuroinvasiveness following intramuscular inoculation was here associated with suppression of multiplication of virus at the site of inoculation. Yet, in the guinea pig, arrest of virus advancement occurred following subcutaneous injection in spite of local multiplication. Thus, it would appear that inhibition of virus growth in a maturing tissue selected for inoculation is but one type of barrier to the progression of virus from the point of inoculation to the next segment.

It should be noted that the change in the clinical picture in the majority of 21-day-old mice injected intramuscularly with the WEE virus is apparently a consequence of the development of a barrier in the blood vessels.

The Coxsackie viruses and the mouse should provide an excellent model for the analysis of the relationships between such aspects as multiplication of virus at the point of inoculation, dissemination, localization in one tissue or organ, etc. The resistance of the growing mouse to these viruses is associated not only with the absence of myositis but also with an inhibition of multiplication of virus in the muscle. The latter can be inferred from the data on the preparation of complement-fixing antigens from infected muscles. Direct evidence of suppression of multiplication at the site of inoculation

was obtained by Hummeler & Sigel (213). They noted that the level of multiplication of Coxsackie virus type H_1 in the inoculated thigh muscles of 12- and 17-day-old mice was 1/10,000 and less than 1/100,000, respectively, of that obtained in three-day-old animals. No inhibitory substance could be demonstrated, however, in the older muscles.

The observations of Pappenehimer *et al.* (117) already cited afford a new point of departure for the exploration of the elements which combine to cause the appearance of different pathologic changes and even different types of illness in animals inoculated at various stages of development with the same virus. To recapitulate, inoculation of the Connecticut type of Coxsackie virus into the muscle or into the brain of adult mice did not lead to the myositis or meningitis usually seen in newborn mice, but instead only to a pancreatitis. At the moment, it is not known why of all the organs only the pancreas remained susceptible.

In a sense, these findings complement those of Sabin & Olitsky. Not only do resistant regions and segments develop in an otherwise susceptible animal, but highly susceptible and vulnerable islands may present themselves within a resistant milieu.

One may ask whether the occurrence of an overt illness or severe disease is invariably associated with a high level of virus multiplication at its point of destination (i.e., the target tissue or organ concerned with a given clinical picture). While it is true that subclinical or mild infections may reflect a low level of virus multiplication (239), it appears that a high level is not a prerequisite for a severe or fatal disease. This can be inferred from the observations of Sabin (239). Working with mice of the PRI strain, he discovered that the resistance of these animals to certain viruses was mediated by a genetic factor inhibiting virus multiplication. Low levels of virus multiplication were found in adult and infant PRI mice inoculated with the 17D strain of yellow fever virus (1/10,000 to 1/100,000 of that obtained in the brains of susceptible mice). Yet, despite this limited multiplication, infant PRI mice succumbed to this virus. Sabin considered this apparent discrepancy a reflection of special cellular vulnerability. This factor, which controls the intensity and the severity of disease, must be distinguished from susceptibility (the ease with which one acquires an infection, regardless of the outcome of the infection). It undoubtedly plays an important role in the different response of young and adult rabbits to the Semliki Forest virus, and perhaps also in the different pathological changes of young and adult chickens infected with Rous sarcoma virus. Previously, Sabin & Olitsky (82) noted a less pronounced neuronal necrosis in older mice as compared with young mice infected with the vesicular stomatitis virus.

Summary.—While our present state of knowledge does not permit a definite correlation between the chemical and physiological changes related to maturation and the changes in susceptibility, there is little doubt that such fundamental mechanisms as metabolism and hormonal influences constitute some of the causes of the observed changes in susceptibility.

Age-induced resistance may be limited to certain regions or certain tis-

sues or it may involve the whole animal. The local changes in susceptibility can cause arrest of virus dissemination.

The regional resistance may appear at different ages varying with the virus, the pathway, and the animal.

Age-induced resistance may reflect an altered cellular response or a modified vulnerability at the final place of localization of the virus or somewhere along its route of travel.

In general, resistance appears to be associated with limited dissemination of the virus and a decreased intensity of cellular damage.

Only through further research on such problems as the mode of virus multiplication, and the mechanics of virus dissemination in a closed system, such as the nerve tracts, and on the meaning of tropism can we expect to obtain an understanding of the influence of age upon susceptibility to virus infections.

AN ATTEMPT AT SYNTHESIS

The changes in susceptibility to virus infections which develop in the course of growth and maturation manifest themselves in the following: the ease of establishment of the virus in the host, the incubation period, the severity of disease, the clinical picture, and the persistence of virus in the host. These changes vary with different host species and different viruses.

The experimental determination of these changes may be affected not only by factors intrinsic in the animal or in a particular strain of virus, but also by extrinsic factors of the experimental procedure. The changes in susceptibility may be sudden or gradual. The direction of these changes is not constant. The differences in the changes, manifestations, and determinations of the influence of age on susceptibility suggest that age exerts its effect through the modification of one or all of the separate elements of the host-virus interaction, such as: resistance of host, tissue, and cell virus invasions, extent of virus multiplication, mode and extent of virus dissemination in the host, and response of cell to infection (degree of vulnerability, ability to recover or tendency to acquire an altered physiology). The modifications which take place in the host may be limited (regional) or general in extent.

In view of the many variations in the influence of age on the susceptibility to virus infections, it is incorrect to apply the term "nonspecific" to age-induced resistance. One should bear in mind that as a result of the modifications induced by growth or maturation, an animal at a given time in its life span may be quite resistant to one virus introduced by one mode of infection and be susceptible to another virus similarly introduced. This animal may be susceptible to the first virus when infected by another route.

LITERATURE CITED

1. Holmes, F. O., *Contribs. Boyce Thompson Inst.*, **4**, 332 (1932)
2. Spizizen, J., *J. Infectious Diseases*, **73**, 212 (1943)
3. Heden, C. G., *Acta Path. Microbiol. Scand.*, Suppl. 88, 1 (1951)

4. Ssacharoff, G. P., *Ergeb. Allgem. Path. u. Path. Anat.*, **22,** 201 (1928)
5. Armstrong, C., *Public Health Repts.*, **45,** 2013 (1930)
6. Leake, J. P., *J. Am. Med. Assoc.*, **101,** 928 (1933)
7. Noka, S., Kingo, S., and Kuroiwa, K., *Fukuoka Acta Med.*, **27,** 30 (1934)
8. Feemster, R. F., *Am. J. Pub. Health*, **28,** 1403 (1938)
9. Leake, J. P., *U. S. Pub. Health Service, Pub. Health Repts.*, **56,** 1902 (1941)
10. Buss, W. C., and Howitt, B. F., *Am. J. Pub. Health*, **31,** 935 (1941)
11. Jackson, F. W., *Am. J. Pub. Health*, **33,** 833 (1943)
12. Getting, V. A., *New Engl. J. Med.*, **224,** 999 (1941)
13. Hammon, W. McD., *J. Am. Med. Assoc.*, **121,** 560 (1943)
14. Hammon, W. McD., *Clinics*, **4,** 485 (1945)
15. Hammon, W. McD., Reeves, W. C., Benner, S. R., and Brookman, B., *J. Am. Med. Assoc.*, **128,** 1133 (1945)
16. Silber, L. A., and Soloviev, V. D., *Am. Rev. Soviet Med.*, Special Suppl. (1946)
17. Warren, J., *Am. J. Trop. Med.*, **26,** 417 (1946)
18. Eklund, C. M., *Am. J. Hyg.*, **43,** 171 (1946)
19. Hauser, G. H., *New Orleans Med. Surg. J.*, **100,** 551 (1948)
20. Panum, P. L., *Observations Made During the Epidemic of Measles on Faroe Islands in Year 1846, Delta Omega Society* (Am. Pub. Health Assoc., New York, N. Y., 1940)
21. Capps, R. B., Bennett, A. M., and Stokes, J., Jr., *Arch. Internal Med.*, **89,** 6 (1952)
22. Burnet, F. M., *Proc. Roy. Australian Coll. Physicians.*, **4,** 95 (1949)
23. Seddon, H. J., Agius, T., Bernstein, H. G. G., and Tunbridge, R. E., *Quart. J. Med.*, **14,** 1 (1945)
24. Nissen, K. I., *Proc. Roy. Soc. Med.*, **40,** 923 (1947)
25. Moses, S. H., *Indian Med. Gaz.*, **83,** 355 (1948)
26. Peart, A. F. W., *Can. Pub. Health J.*, **40,** 405 (1949)
27. Sigurgonsson, J., *Am. J. Hyg.*, **51,** 109 (1950)
28. Su, T. L., *Indian Med. Gaz.*, **79,** 332 (1944)
29. Macchiavello, A., and Cifuentes, O., *Rev. chilena hig. y med. prevent.*, **7,** 15 (1915)
30. Bingham, E. M., *Calif. Med.*, **65,** 225 (1946)
31. Hammon, W. M., *Bact. Revs.*, **13,** 135 (1949)
32. Sabin, A. B., *Am. J. Pub. Health*, **41,** 1215 (1951)
33. Greene, H. S. N., *J. Exptl. Med.*, **62,** 305 (1935)
34. Rosahn, P. D., and Hu, C. K., *J. Exptl. Med.*, **62,** 331 (1935)
35. Greene, H. S. N., *J. Exptl. Med.*, **61,** 807 (1935)
36. Beach, J. R., *Science*, **100,** 361 (1944)
37. Fabricant, J., *Cornell Vet.*, **40,** 39 (1950)
38. Traub, E., *J. Exptl. Med.*, **63,** 533 (1936)
39. Traub, E., *J. Exptl. Med.*, **64,** 183 (1936)
40. Traub, E., *J. Exptl. Med.*, **68,** 229 (1938)
41. Whitney, E., *Proc. Soc. Exptl. Biol. Med.*, **78,** 247 (1951)
42. Traub, E., *J. Exptl. Med.*, **69,** 801 (1939)
43. Haas, V. H., *U. S. Pub. Health Service, Pub. Health Repts.*, **56,** 285 (1941)
44. Gregg, N. McA., *Trans. Ophthalmol. Soc. Australia*, **3,** 35 (1942)
45. Swan, C., Tostevin, A. L., Moore, B., Mayo, H., and Barham-Black, G. H., *Med. J. Australia*, **2,** 201 (1943)
46. Aycock, W. L., and Ingalls, T. H., *Am. J. Med. Sci.*, **212,** 366 (1946)

47. Reese, A. B., *Am. J. Ophthalmology*, **27**, 483 (1944)
48. Erickson, C. A., *J. Pediat.*, **25**, 281 (1944)
49. Swan, C., Tostevin, A. L., and Barham-Black, G. H., *Med. J. Australia*, **2**, 889 (1946)
50. Miller, H. C., *Pediatrics*, **5**, 320 (1950)
51. Lynch, F. W., *Arch. Dermatol. u. Syphilis*, **26**, 997 (1932)
52. Hamburger, V., and Habel, K., *Proc. Soc. Exptl. Biol. Med.*, **66**, 608 (1947)
53. Markham, F. S., and Hudson, N. P., *Am. J. Path.*, **12**, 175 (1936)
54. Stritar, J., and Hudson, N. P., *Am. J. Path.*, **12**, 165 (1936)
55. Woolpert, O. C., Gallagher, F. W., Rubinstein, L., and Hudson, N. P., *J. Exptl. Med.*, **68**, 313 (1938)
56. Dettwiler, H. A., Hudson, N. P., and Woolpert, O. C., *J. Exptl. Med.*, **72**, 623 (1940)
57. Woolpert, O. C., *Am. J. Path.*, **12**, 141 (1936)
58. Beveridge, W. I. B., and Burnet, F. M., *Med. Research Council (Brit.), Special Rept. Ser.*, No. 256 (1946)
59. Sigel, M. M., *Abstracts of Doctoral Dissertations*, No. 44, 187 (The Ohio State University Press, Columbus, Ohio, 1944)
60. Bang, F. B., *J. Exptl. Med.*, **76**, 263 (1942)
61. Bang, F. B., *J. Exptl. Med.*, **77**, 337 (1943)
62. Fox, J. P., and Laemmert, H. W., Jr., *Am. J. Hyg.*, **46**, 21 (1947)
63. Sigurdsson, B., *J. Exptl. Med.*, **78**, 17 (1943)
64. Koprowski, H., and Cox, H. R., *J. Immunol.*, **60**, 533 (1948)
65. Bernkopf, H., and Kligler, I. J., *Proc. Soc. Exptl. Biol. Med.*, **45**, 332 (1940)
66. Schlesinger, R. W., *Proc. Soc. Exptl. Biol. Med.*, **76**, 817 (1951)
67. Crawley, J. F., *J. Immunol.*, **59**, 83 (1948)
68. Enders, J. F., and Pearson, H. E., *Proc. Soc. Exptl. Biol. Med.*, **48**, 143 (1941)
69. Chamberlain, R. W., and Kissling, R. E. (Personal communication)
70. Miller, G. L., *J. Exptl. Med.*, **79**, 173 (1944)
71. vonMagnus, P., *Acta Path. Microbiol. Scand.*, **28**, 250 (1951)
72. Hirst, G. K., *Proc. Soc. Exptl. Biol. Med.*, **58**, 155 (1945)
73. Burnet, F. M., Beveridge, W. I. B., and Bull, D. R., *Australian J. Exptl. Biol. Med. Sci.*, **22**, 9 (1944)
74. Koprowski, H., and Cox, H. R., *J. Immunol.*, **52**, 171 (1946)
75. Andervont, H. B., *Am. J. Hyg.*, **7**, 804 (1927)
76. Andervont, H. B., *J. Infectious Diseases*, **44**, 383 (1929)
77. Theiler, M., *Ann. Trop. Med. Parasitol.*, **24**, 249 (1930)
78. International Committee for the Study of Infantile Paralysis, *Poliomyelitis* (Williams & Wilkins Co., Baltimore, Md., 562 pp., 1932)
79. Olitsky, P. K., Sabin, A. B., and Cox, H. R., *J. Exptl. Med.*, **64**, 723 (1936)
80. Sabin, A. B., and Olitsky, P. K., *J. Exptl. Med.*, **66**, 15 (1937)
81. Sabin, A. B., and Olitsky, P. K., *J. Exptl. Med.*, **66**, 35 (1937)
82. Sabin, A. B., and Olitsky, P. K., *J. Exptl. Med.*, **67**, 201 (1938)
83. Sabin, A. B., and Olitsky, P. K., *J. Exptl. Med.*, **67**, 229 (1938)
84. Sabin, A. B., and Olitsky, P. K., *Proc. Soc. Exptl. Biol. Med.*, **38**, 595 (1938)
85. Sabin, A. B., and Olitsky, P. K., *Proc. Soc. Exptl. Biol. Med.*, **38**, 597 (1938)
86. O'Leary, J. L., Smith, M. G., and Reames, H. R., *J. Exptl. Med.*, **75**, 233 (1942)
87. Lennette, E. H., and Koprowski, H., *J. Immunol.*, **49**, 175 (1944)
88. King, L. S., *J. Exptl. Med.*, **72**, 573 (1940)
89. Melnick, J. L., Paul, J. R., Riordan, J. T., Barnett, V. H., Goldblum, N., and Zabin, E., *Proc. Soc. Exptl. Biol. Med.*, **77**, 661 (1951)

90. Kilbourne, E. D., and Horsfall, F. L., Jr., *J. Immunol.*, **67**, 321 (1951)
91. Casals, J., *J. Exptl. Med.*, **72**, 445 (1940)
92. King, L. S., *J. Exptl. Med.*, **71**, 95 & 107 (1940)
93. Kissling, R. E. (Unpublished data)
94. Bugher, J. C., *Am. J. Trop. Med.*, **21**, 299 (1941)
95. Howitt, B. F., *J. Infectious Diseases*, **67**, 177 (1940)
96. Watson, D. W., and Smadel, J. E., *Proc. Soc. Exptl. Biol. Med.*, **52**, 101 (1943)
97. Meyer, K. F., *Ann. Internal Med.*, **6**, 645 (1932)
98. Meyer, K. F. (Personal communication)
99. Schlesinger, R. W., Morgan, I. M., and Olitsky, P. K., *J. Am. Med. Assoc.*, **119**, 618 (1942)
100. Sellards, A. W., Tyzzer, E. E., and Bennett, B. L., *Am. J. Hyg.*, **33**, 63 (1941)
101. Duffy, C. E., and Sabin, A. B., *J. Bact.*, **43**, 88 (1942)
102. Duffy, C. E., *Proc. Soc. Exptl. Biol. Med.*, **76**, 566 (1951)
103. Olitsky, P. K., *Proc. Soc. Exptl. Biol. Med.*, **43**, 296 (1940)
104. Olitsky, P. K., *J. Exptl. Med.*, **72**, 113 (1950)
105. Theiler, M., and Gard, S., *J. Exptl. Med.*, **72**, 49 (1940)
106. Theiler, M., and Gard, S., *J. Exptl. Med.*, **72**, 79 (1940)
107. vonMagnus, H., *Acta Path. Microbiol. Scand.*, **29**, 251 (1951)
108. vonMagnus, H., and vonMagnus, P., *Acta Path. Microbiol. Scand.*, **26**, 175 (1949)
109. Mandel, B., *Bull. N. Y. Acad. Med.*, **27**, 583 (1951)
110. vonMagnus, H., *Acta Path. Microbiol. Scand.*, **27**, 611 (1950)
111. Sabin, A. B. (Personal communication)
112. Dalldorf, G., and Sickles, G. M., *Science*, **108**, 61 (1948)
113. Melnick, J. L., Shaw, E. W., and Curnan, E. C., *Proc. Soc. Exptl. Biol. Med.*, **71**, 344 (1949)
114. Dalldorf, G., *Bull. N. Y. Acad. Med.*, **26**, 329 (1950)
115. Kaplan, A. S., and Melnick, J. L., *Proc. Soc. Exptl. Biol. Med.*, **76**, 312 (1951)
116. Goldblum, N. (Personal communication)
117. Pappenheimer, A. M., Kunz, L. J., and Richardson, S., *J. Exptl. Med.*, **94**, 45 (1951)
118. Duran-Reynals, F., *Yale J. Biol. and Med.*, **13**, 77 (1940–41)
119. Duran-Reynals, F., *Cancer Research*, **2**, 343 (1942)
120. Duran-Reynals, F., and King, J. W., *Cancer Research*, **7**, 21 (1947)
121. Duran-Reynals, F., *Yale J. Biol. and Med.*, **22**, 555 (1949)
122. Blakemore, F., *J. Comp. Path. Therap.*, **52**, 114 (1939)
123. Duran-Reynals, F., *Yale J. Biol. and Med.*, **13**, 99 (1940–41)
124. Duran-Reynals, F., *Cancer Research*, **5**, 25 (1945)
125. Bittner, J. J., *Cancer Research*, **2**, 540 (1942)
126. Gross, L., *Proc. Soc. Exptl. Biol. Med.*, **78**, 342 (1951)
127. Duran-Reynals, F., *Science*, **103**, 748 (1946)
128. Kalter, S. S., *J. Immunol.*, **63**, 17 (1949)
129. Young, G. A. (Personal communication)
130. Fenner, F., *Australian J. Exptl. Biol. Med. Sci.*, **27**, 45 (1949)
131. Pinto, M. R., *Am. J. Hyg.*, **48**, 361 (1948)
132. Sabin, A. B., *Proc. Soc. Exptl. Biol. Med.*, **73**, 394 (1950)
133. Dalldorf, G., *The Pathogenesis and Pathology of Viral Diseases* (Columbia Univ. Press, New York, N. Y., 235 pp., 1950)
134. Casals, J., Olitsky, P. K., and Anslow, R. O., *J. Exptl. Med.*, **94**, 111 (1951)
135. Casals, J., and Olitsky, P. K., *Arch. ges. Virusforsch.*, **4**, 452 (1951)

136. Hammon, W. McD., Cheever, F. S., and Sather, G. E., *Proc. Soc. Exptl. Biol. Med.*, **78,** 293 (1951)
137. Olitsky, P. K., and Harford, C. G., *J. Exptl. Med.*, **68,** 173 (1938)
138. Whitman, L., *Am. J. Trop. Med.*, **23,** 17 (1943)
139. Lennette, E. H., and Koprowski, H., *J. Immunol.*, **49,** 375 (1944)
140. Lennette, E. H., and Koprowski, H., *J. Immunol.*, **52,** 343 (1946)
141. von Magnus, H., *Acta Path. Microbiol. Scand.*, **29,** 243 (1951)
142. Rustigian, R., and Pappenheimer, A. M., *J. Exptl. Med.*, **89,** 69 (1949)
143. Oliphant, J. W., and Tibbs, R. O., *U. S. Pub. Health Service, Pub. Health Repts.*, **65,** 521 (1950)
144. Huebner, R. J., Cole, R. M., Beeman, E. A., Bell, J. A., and Peers, J. H., *J. Am. Med. Assoc.*, **145,** 628 (1951)
145. Weller, T. H., Enders, J. F., Buckingham, M., and Finn, J. J., *J. Immunol.*, **65,** 337 (1950)
146. Melnick, J. L., and Kraft, L. M., *Federation Proc.*, **9,** 585 (1950)
147. Perla, D., and Marmoston, J., *Natural Resistance and Clinical Medicine* (Little, Brown & Co., Boston, Mass., 1344 pp., 1941)
148. Burnet, F. M., *Biological Aspects of Infectious Disease* (Cambridge Univ. Press, Cambridge, England, 308 pp., 1940)
149. Shock, N. W., *A Classified Bibliography of Gerontology and Geriatrics* (Stanford Univ. Press, Stanford, Calif., 599 pp., 1948)
150. Benedict, F. G., and MacLeod, G., *J. Nutrition*, **1,** 367 (1929)
151. Black, A., and Murlin, J. R., *J. Nutrition*, **17,** 347 (1939)
152. Himwich, H. E., *Brain Metabolism and Cerebral Disorders* (Williams and Wilkins Co., Baltimore, Md., 451 pp., 1951)
153. Tyler, D. B., and van Harreveld, A., *Am. J. Physiol.*, **136,** 600 (1942)
154. Welsh, J. H., and Hyde, J. E., *J. Neurophysiol.*, **7,** 41 (1944)
155. Lazovskaya, L. N., *Biokhimiya*, **8,** 171 (1943)
156. Murray, A. Z., Greenstein, L. M., and Sherman, H. C., *J. Biol. Chem.*, **165,** 91 (1946)
157. Cheymal, J., and Pelon, A., *Compt. rend. soc. biol.*, **138,** 91 (1944)
158. Boy, G., and Cheymal, J., *Bull. soc. chim. biol.*, **27,** 105 (1945)
159. Boy, G., Cheymal, J., and Pelon, A., *Bull. soc. chim. biol.*, **27,** 167 (1945)
160. Moog, F., *J. Exptl. Zoöl.*, **105,** 209 (1947)
161. Henry, K. M., and Kon, S. K., *Biochem. J.*, **41,** 169 (1947)
162. Gobat, Y., *Helv. Med. Acta*, **14,** 45 (1947)
163. Horvath, S. M., *Am. J. Physiol.*, **145,** 77 (1945)
164. Falkenheim, M., *Am. J. Physiol.*, **138,** 175 (1942)
165. Burroughs, E. W., Burroughs, H. S., and Mitchell, H. H., *J. Nutrition*, **19,** 363 (1940)
166. Cutting, M., and McCance, R. A., *J. Physiol., (London)*, **105,** 205 (1946)
167. Pearce, J. M., *Am. J. Physiol.*, **114,** 255 (1936)
168. Raschba, J. J., *Chem. Zentr.*, **113,** 3226 (1942)
169. Cole, V. V., and Koch, F. C., *J. Biol. Chem.*, **94,** 263 (1931–32)
170. Reiner, J. M., *J. Gerontol.*, **2,** 315 (1947)
171. Nickle, M., and Kabat, H., *J. Exptl. Med.*, **80,** 247 (1944)
172. Wood, H. G., Rusoff, I. I., and Reiner, J. M., *J. Exptl. Med.*, **81,** 151 (1945)
173. Racker, E., and Krimsky, K., *J. Exptl. Med.*, **84,** 191 (1946)
174. Potter, V. R., Schneider, W. C., and Liebl, G. J., *Cancer Research*, **5,** 21 (1945)

175. Conference on The Chick Embryo in Biological Research, *Ann. N. Y. Acad. Sci.* (In press)
176. Fox, J. P., *Am. J. Hyg.*, **46**, 1 (1947)
177. Pearson, H. E., *J. Immunol.*, **64**, 447 (1950)
178. Howe, H. A., *The Pathogenesis and Pathology of Viral Diseases*, 214 (Columbia University Press, New York, N. Y., 235 pp., 1950)
179. Greiff, D., *The Rickettsial Diseases of Man*, 51 (American Association for Advancement of Science, Washington, D. C., 247 pp., 1948)
180. Rafelson, M. E., Jr., Winzler, R. J., and Pearson, H. E., *J. Biol. Chem.*, **181**, 583 (1949)
181. Anderson, J. A., Gemzell, C., Gemzell, L., Bolin, V. S., and Samuels, L. T., *Proc. Soc. Exptl. Biol. Med.*, **73**, 690 (1950)
182. Rafelson, M. E., Jr., Pearson, H. E., and Winzler, R. J., *Arch Biochem.*, **29**, 69 (1950)
183. Rafelson, M. E., Jr., Winzler, R. J., and Pearson, H. E., *J. Biol. Chem.*, **193**, 205 (1951)
184. Block, R. J., *J. Biol. Chem.*, **120**, 467 (1937)
185. Donaldson, H. H., *Mem. Wistar Inst. Anat. Biol.*, No. 6, 278 pp., (1924)
189. Flexner, J. B., Flexner, L. B., and Straus, W. L., *J. Cellular Comp. Physiol.*, **18**, 355 (1941)
190. Kuntz, A., *Am. J. Path.*, **14**, 783 (1938)
191. Cottrell, L., *Arch. Neurol. Psychiat.*, **43**, 1138 (1940)
192. Lassek, A. M., and Rasmussen, G. L., *J. Comp. Neurol.*, **69**, 371 (1938)
193. Andrew, W., *Cellular Changes With Age*, Charles C Thomas, Publisher, Springfield, Ill., 74 pp., 1952)
194. Andrew, W., *Am. J. Physiol.*, **119**, 260 (1937)
195. Biesele, J. J., *J. Gerontol.*, **1**, 433 (1946)
196. Foley, E. J., *J. Bact.*, **43**, 776 (1942)
197. Pearce, L., Rosahn, P. D., and Hu, C. K., *Proc. Soc. Exptl. Biol. Med.*, **31**, 657 (1933–34)
198. Ørskov, J., and Andersen, E. K., *Z. Immunitätsforsch.*, **92**, 477 (1938)
199. Freund, J., *J. Exptl. Med.*, **54**, 171 (1931)
200. Sawyer, W. A., and Lloyd, W., *J. Exptl. Med.*, **54**, 533 (1931)
201. Hurst, E. W., *J. Comp. Path. Therap.*, **60**, 237 (1950)
202. Baumgartner, L., *Yale J. Biol. and Med.*, **6**, 403 (1933–34)
203. Morgan, I. M., *Proc. Soc. Exptl. Biol. Med.*, **44**, 473 (1940)
204. Morgan, I. M., *J. Exptl. Med.*, **74**, 115 (1941)
205. Casals, J., *J. Exptl. Med.*, **72**, 453 (1940)
206. Duran-Reynals, F., *Yale J. Biol. and Med.*, **13**, 61 (1940–41)
207. Duran-Reynals, F., *Yale J. Biol. and Med.*, **13**, 693 (1941)
208. Curley, F. J., and Aycock, W. L., *Endocrinology*, **39**, 414 (1946)
209. Foley, G. E., and Aycock, W. L., *Endocrinology*, **37**, 245 (1945)
210. Kalter, S. S., Stuart, D. C., and Tepperman, J., *Proc. Soc. Exptl. Biol. Med.*, **74**, 605 (1950)
211. Kilbourne, E. D., and Horsfall, F. L., Jr., *Proc. Soc. Exptl. Biol. Med.*, **77**, 135 (1951)
212. Kilbourne, E. D., and Horsfall, F. L., Jr., *Proc. Soc. Exptl. Biol. Med.*, **76**, 116 (1951)
213. Hummeler, K., and Sigel, M. M. (Unpublished data)

214. Schwartzman, G., *Proc. Soc. Exptl. Biol. Med.*, **75,** 835 (1951)
215. Smith, J. M., Murphy, J. S., and Mirick, G. S., *Proc. Soc. Exptl. Biol. Med.*, **78,** 505 (1951)
216. Foster, C., Sigel, M. M., Henle, W., and Stokes, J., Jr., *J. Lab. Clin. Med.*, **38,** 359 (1951)
217. Ainslie, J. D., Francis, T., Jr., and Brown, G. C., *J. Lab. Clin. Med.*, **38,** 344 (1951)
218. Parker, R. E., *Science*, **74,** 181 (1931)
219. Baker, L. E., and Carrel, A., *J. Exptl. Med.*, **45,** 305 (1927)
220. Casals, J., and Olitsky, P. K., *Science*, **106,** 267 (1947)
221. Williams, H. H., Galbraith, H., Kaucher, M., Moyer, E. Z., Richards, A. J., and Macy, I. G., *J. Biol. Chem.*, **161,** 475 (1945)
222. Shope, R. E., *J. Biol. Chem.*, **80,** 141 (1928)
223. Folch, J., Casals, J., Meath, J. A., and Pope, A. (Personal communication)
224. Dozois, T. F., Wagner, J. C., Chemerda, C. M., and Andrew, V. M., *J. Immunol.*, **62,** 319 (1949)
225. Moro, E., *Uber das Verhalten Haemolytischer Serumstoffe beim Gesunden und Krankenkind* (J. F. Bergmann, Wiesbaden, Germany, 100 pp., 1908)
226. King, J. W., and Duran-Reynals, F., *Yale J. Biol. and Med.*, **16,** 53 (1943–44)
227. Laemmert, H. W., and Moussatche, J., *J. Infectious Diseases*, **72,** 229 (1943)
228. Schlesinger, R. W., and Frankel, J. W., *Am. J. Trop. Med. Hyg.*, **1,** 66 (1952)
229. Overman, J. R., and Dortch, H., Jr., *Am. J. Path.*, **27,** 1065 (1951)
230. Sabin, A. B., *J. Pediat.*, **19,** 596 (1941)
231. Sabin, A. B., and Duffy, C. E., *Science*, **91,** 552 (1940)
232. Pinkerton, H., and Bessey, O. A., *Science*, **89,** 368 (1939)
233. Rasmussen, A. F., Jr., Waisman, H. A., and Lichstein, H. C., *Proc. Soc. Exptl. Biol. Med.*, **57,** 92 (1944)
234. Kearney, E. B., Pond, W. L., Plass, B. A., Maddy, K. H., Elvehjem, C. A., and Clark, P. F., *J. Infectious Diseases*, **82,** 177 (1948)
235. Davies, W. L., Smith, S. C., Pond, W. L., Rasmussen, A. F., Jr., and Clark, P. F., *Proc. Soc. Exptl. Biol. Med.*, **72,** 528 (1949)
236. Bodian, D., *Am. J. Hyg.*, **48,** 87 (1948)
237. Jones, J. H., Foster, C., and Henle, W., *Proc. Soc. Exptl. Biol. Med.*, **69,** 454 (1948)
238. Langworthy, O. R., *Carnegie Inst. Wash.*, **24,** 3 (1933)
239. Sabin, A. B., *Ann. N. Y. Acad. Sci.* (1951)
240. Kempe, C. H., Benenson, A. S., and Jackson, J. R., *Federation Proc.*, **11,** 473 (1952)
241. Zinsser, H., and Schoenbach, E. B., *J. Exptl. Med.*, **66,** 207 (1937)
242. Plotz, H., *Compt. rend. soc. biol.*, **125,** 603 (1937)
243. Plotz, H., *Compt. rend. soc. biol.*, **125,** 719 (1937)

TOXOPLASMA AND TOXOPLASMOSIS[1]

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Toxoplasma

Extraordinary interest in toxoplasmosis has been shown in the last decade, following the demonstration that it is a human pathogen lethal for infants and for adults. Medically, it gave the challenge of recognition, description, and treatment—a task by no means completed. Biologically, it added a potent anthropocentric stimulus to the investigation of a little understood microorganism.

Toxoplasma infection in man results in abortions, miscarriages, stillbirths, and, in infants who survive, various intellectual and physical defects. These infections are contracted from the female parent, who is almost invariably in good health. In these congenital infections, the brain, eye, and spinal cord bear the brunt of the disease, and both the clinical and pathological pictures seem outlined. Fatal cases in adults show a less uniform pattern —the central nervous system is often not conspicuously involved, and predominant manifestations vary according to the tissue localization of the microörganism, which is remarkably unrestricted. Finally, it is also known that infection may exist without symptoms, and is revealed, when the healthy individual is a pregnant female, by the birth of an infected child. The prevalence of human infections is unknown; to be evaluated are reports of reactions to toxoplasma antigen by 30 to 60 per cent of the normal adult urban population.

In 1944 the author published a review of the field (104), and the present paper is based chiefly on subsequent publications, of which over 300 have appeared. Monographic treatment is beyond present space limitations and will be published elsewhere.[2] It is the intent of this article to summarize present knowledge of the organism and to discuss selected features of interest which have been investigated in its human and other animal hosts. Emphasis will be placed on recent developments; however, most of the basic problems of 1944 remain unsolved.

Characterization.—Toxoplasma is nearly unique among protozoa in its lack of specificity. It occurs naturally in a great variety of mammals and in warm-blooded vertebrates. In this huge group, comprising essentially all the

[1] The survey of the literature pertaining to this review was concluded in February, 1952.

[2] The excellent monograph by Binkhorst, published in 1948 (2) contains an extensive bibliography, as does the article by Biocca (3). Medical and other aspects were reviewed by Callahan *et al.* in 1946 (11). Excellent detailed pathological descriptions are given in the papers of Wolf, Cowen & Paige (e.g., 114, 115).

homeotherms, no proved case of insusceptibility to experimental infection has been encountered.[3]

This lack of specificity applies at the cellular level also, viewed not only as to derivation, since cells originating from all three germ layers are invaded; but also in regard to differentiation, highly specialized cells of very different types proving suitable for multiplication (104).

Toxoplasma is an obligate parasite so far as is known, and has not yet been cultivated either extracellularly or on cell-free media. Cellular localization is always cytoplasmic, never intranuclear (104). Reproduction is by binary longitudinal fission; verification of schizogony (12) has not been obtained. In infected mammals, toxoplasma gives rise to antibodies which are protective (86), fix complement (68), are stain-inhibiting (85), and reduce egg pock-counts (52). It also elicits skin reactions (32, 34) and is involved in the formation of a potent toxin *in vivo* (107).

The distribution is world-wide, and Toxoplasma has been reported from the tropics (112) and to about 55° North Latitude (13).

Classification.—Toxoplasma is probably a protozoön; notably all efforts to discover fungal characteristics have failed. *Toxoplasma gondii* Nicolle and Manceaux, 1909, is the type species and at present the only valid one. All toxoplasmata, of whatever isolation, are morphologically indistinguishable and infect the same range of animals and cross-immunize [Mesnil (58); Wolf, Cowen & Paige (115, 116); Sabin (83)].

Affinities of the organism are not clear [Brumpt (10)]. It was placed with other intracellular protozoa without locomotor organs in the class Sporozoa (28, 29) on the basis of characteristics not since confirmed. This is now a procrustean classification and has neither the advantage of a mnemonic device nor of indicating relationships.

Morphology.—Single extracellular organisms are usually crescentic in the fresh state and measure about $6 \times 2\frac{1}{2}\mu$. One end is more blunt than the other, and the nucleus is in this portion. The cytoplasm occasionally contains one or more granules but no blepharoplast, nor any other structure of constant occurrence. There is a distinct cell membrane, for after treatment causing extrusion of the cell contents, the cell membrane is left behind (103). Nuclear chromatin takes the usual stains and gives the Feulgen reaction (82, 19, 51). This constitutes no evidence at all against the fungal nature of the organism since Feulgen-positive pathogenic fungi are well known (50). No chromatin is demonstrable on the nuclear membrane by usual cytologic techniques, but the statement that there is no nuclear membrane has not been confirmed (19). There is no organ of locomotion and descriptions of flagella probably have another explanation. Slow twisting and rocking motion may be observed at 37°C., less often at lower temperatures (104). Organisms which have just divided separate with great rapidity at times

[3] Experimental infection of heterotherms has failed [Manwell *et al.* (56, 47b)]; consequently, records of spontaneous infections require verification.

(103), probably because of physico-chemical factors rather than true motility. The active serpentine movements of *Sarcocystis* do not occur (103), and there is no formation of pseudopodia. The typical crescentic shape is well retained in smears rapidly dried or fixed, but after standing, after antibody action (85), or after tissue fixation the organisms become shorter and broader and then assume an ovoid outline.

Within cells the organisms divide repeatedly. In syncytial tissue, such as striated and heart muscle, huge spindle-shaped collections may be seen, numbering several hundred organisms (75). Such collections do not have a "cyst" membrane (103) and may be seen passing from one fiber to another. In nonsyncytial cells, repeated divisions lead first to flattening, then to granulation of the host-cell nucleus (19) and the cytoplasmic remnants are reduced to a fine thin membrane. The whole then resembles a protozoön cyst, and hence is termed a pseudocyst. The limiting membrane of the pseudocyst has a double contour in fresh preparations (79), stains by silver impregnation methods (33), and following Van Gieson's stain stands out sharply (47). When pseudocysts are crushed they liberate typical single organisms, and they leave behind in the ruptured cyst a channeled ground substance which is probably the residue of the cell cytoplasm (103). These formations are really cyst-like in their resistance to mechanical shock, for pseudocysts may be isolated from brain tissue by shaking with glass beads (79), but it is important to realize that *Toxoplasma* produces no cyst wall; accordingly, the limiting membrane will be present or absent according to the tissue in which the parasite is situated. "Terminal colony" has also been used to designate the intracellular formations (19).

A detailed cytological study of *Toxoplasma* describes the "cytostyle" as "a shadowy thickened band of cytoplasm [which] divides the parasite longitudinally and lies excentrically, just beneath the cell membrane" [Cross (19)]. This has been questioned as fixation artefact, but what corresponds to the above-described structure was seen in living material with the phase microscope (103). It is probably an inconstant morphological feature, possibly produced by the bending and twisting movements of the the organism. The "phlange"—a clear area in the concavity of the parasite —has also been reported (19). Cross (19) describes the stages of mitosis in extracellular forms, the nuclear membrane being retained throughout. The majority of intracellular forms were in prophase. Although a spindle was described earlier (77), confirmation has not been obtained (19).

In pseudocysts, nuclei are stained in all specimens by the Feulgen technique but give irregular results in the Bauer reaction. The polysaccharides responsible for the Bauer reaction are digested slowly by diastase [Lillie (49a, 51)]. Tests for "volutin" have been negative, whereas fat staining with Sudan 4 but not with osmium tetroxide in Flemming's fluid has been demonstrated (19).

Life cycle.—The organism enters both phagocytic and nonphagocytic cells and divides repeatedly by binary longitudinal fission. If the host

cell ruptures, organisms are freed to enter new cells and repeat their development. This is the only type of cycle known at present. It is assumed that extracellular organisms have been liberated from cells.

Schizogony has not been confirmed. Supporting data fall into two classes: in one case they are unconvincing [see for example (9)] and in the second case true schizogony is indeed suggested, but the relationship to *Toxoplasma* is indefinite, since the findings have been made in trapped wild animals [Chatton & Blanc (12)].

The division rate is once every six hours during the first 24 (20). Toxoplasmata injected intraperitoneally into mice are not found eight hours later. A few then appear during the first 24 hours, and there is a steep rise in numbers from 24–48 hours with a further rise from 48–72 hours, although the rate of increase falls off (20).

Differentiation.—Other microörganisms encountered in tissues may have to be distinguished from *Toxoplasma*. Features allowing discrimination between *Toxoplasma* and tissue forms of *Trypansosoma cruzi*, *Leishmania* sp., *Histoplasma*, and *Sarcocystis* have been given (74, 104).

Encephalitozoon, at one time confused with *Toxoplasma*, is undoubtedly a different organism, as the majority of workers who have had the two at their disposal now agree. Recent statements that the two are identical (89, 3) ignore the following evidence, which comes chiefly from Perrin: (*a*) The two are different in size, in shape, and in staining reactions (67, 72, 104, 65). (*b*) Cellular localization shows many differences. (*c*) *Encephalitozoon* does not produce an acute fatal disease in any animal. (*d*) *Encephalitozoon* can be preserved in 50 per cent buffered glycerin or when frozen at −70°C. while *Toxoplasma* dies rapidly under these conditions (72). (*e*) *Encephalitozoon* does not immunize against *Toxoplasma* (104).

Epidemiological investigations in birds should be evaluated with the knowledge that other organisms have been mistaken for *Toxoplasma*. At least three have been recognized: exoerythrocytic forms of bird malaria, structures termed X-bodies, and the ineptly named "avian *Toxoplasma*" (56, 57). None of the three infect mammals, whereas *Toxoplasma* readily infects mice or guinea pigs, whether isolated from pigeons (44, 112) or passed repeatedly in chick embryos (53).

Through the courtesy of Dr. J. K. Frenkel, information has been obtained about a microörganism resembling *Toxoplasma* discovered in wild *Peromyscus* and referred to provisionally as the "P organism." Individual organisms are said to resemble *Toxoplasma* very closely in size and shape; the staining reactions with the periodic Schiff technique are different. The major distinguishing feature is the formation of heavy-walled cysts with cytoplasm containing nuclei applied to the inner surface. The organism is inoculable to other rodents, and the susceptibility of primates is unknown. The P organism does not cross-immunize with *Toxoplasma*, and infected animals do not give a positive dye reaction in the *Toxoplasma* test (30).

Physiology.—Toxotoxin, a potent toxin associated with *Toxoplasma*,

will be described later. Toxoplasma multiplies within nerve cells of the brain and spinal cord, in cells of the retina, within myocardial fibers, in hepatic cells, within motile phagocytes and reticulo-endothelial cells, inside vascular endothelial cells not considered to be phagocytic, in fat cells, connective tissue cells, and so forth. Toxoplasma does not occur within red cells, one such observation (116) not having been confirmed, but it does circulate in the blood stream (43, 93). Little is known about metabolism and growth factors. Although toxoplasma has not been cultivated *in vitro*, it is possible to obtain suspensions in peritoneal fluid, relatively free of host cells, and to maintain organisms alive at 37°C. for periods which should be adequate for manometric studies.

Unusual resistance to physical agents and common antiseptics has not been demonstrated. Toxoplasmosis may be contracted by feeding, but this is not because of a marked resistance to peptic digestion, for the organism is not infectious after exposure to artificial gastric juice mixtures used to digest viable trichinae free from muscle (103).

In finely ground tissue, presumably not containing pseudocysts, toxoplasmata were killed in 10 min. with two volumes of 5 per cent phenol or 70 per cent alcohol (56). Tabulated data on the effect of chemotherapeutic agents *in vitro* are available elsewhere (88).

Rather widely different results are given for survival at temperatures between 3° and 5°C.: 8 days (23), 14 days (88) and 30 days (53). *Toxoplasma* survives freezing and lowering of temperature to −70°C. (108). In fact, it was not possible to destroy the infectivity of a tissue suspension by as many as six rapid freezings (at −70°C.) and thawings. Storage at −70°C. for any prolonged period however, is not successful. This fact is unusual, since protozoa usually die during the freezing process or subsequent lowering of temperature, but if they survive this, they can be stored for long periods at −70° or below (108). Infectivity of peritoneal exudate is lost after 15 min. at 50°C. or 5 min. at 55°C. (57).

Efforts to ascribe to *Toxoplasma* a pronounced neurotropism (36) or predominant, if not exclusive, localization in the reticulo-endothelial system (20) ignore the unusual "pantropism" of this organism which has already been described. The susceptibility of brain tissue to small inocula will be discussed later.

Culture.—*Toxoplasma* multiplies in the chick embryo (48, 101), the mouse embryo (14, 22), and tissue cultures (49, 86), but has not yet been grown in the absence of living cells. It has failed to grow extracellularly in association with *Trypanosoma cruzi* (103), or to develop in the pooled embryonic fluid of 17-day fertile eggs (53).

Toxin.—Toxotoxin is a potent toxin with unusual properties which develops in animals infected with *Toxoplasma* [Weinman & Klatchko (107)]. It occurs in the ascitic fluid of intraperitoneally injected mice. Injected intravenously into mice, the fluid is lethal with extreme rapidity, one MLD calculated to the nearest 0.1 cc. usually killing within 1 to 3 min. Samples of

different ascitic fluids reveal MLDs varying from 0.1 to 0.5 cc. for a 20-gm. mouse. Mice tolerate ascitic fluids produced by other means when injected intravenously in amounts up to 0.7 cc. This tolerance extends to ascitic fluids produced with chemicals (lactic acid), killed bacteria (streptococci) and a cancerous intraperitoneal graft,[4] and to hamster ascitic fluid caused by infection with *Leishmania donovani*. Normal mouse serum stored at 37°C. with or without added tissue is also innocuous (103).

Toxotoxin may prove to have more than one component. Whether it is an endotoxin, an exotoxin, or a reaction product between organism and host remains to be determined. There is so far no evidence that it is an endotoxin; doses of 6×10^7 organisms, first washed, then repeatedly frozen and thawed, have not produced immediate toxic symptoms. Furthermore, toxic fluid stored at 8°C. with organisms has not changed detectably in titer. Thermostability is an outstanding characteristic of the substance. Toxicity is not decreased appreciably by storage for 20 days at 5°C. nor by heating in a boiling water bath for a half-hour. Activity is reduced, however, by autoclaving at 15 lb. pressure (121°C.) for 20 min. Conversely, in specimens stored for months, activity has actually been increased by mild heat (56°C. for 30 min.).

Toxicity is not destroyed by ultraviolet irradiation (8 min., 2537 Å approximate delivered intensity 346 microwatts/cm²). Incubation with neutral formalin to 0.1 per cent final concentration for four hours at 37°C. effected no detectable change; with 0.3 per cent formalin for 30 days at 37°C. the titer was reduced about one-half.

Since the toxin is produced *in vivo* and is not an artifice of preparation, it may well be implicated in the genesis of symptoms. The site and mode of action have not been determined, although symptoms suggest respiratory distress and central nervous system attack. Mice have not been protected by nikethamide (coramine), atropine, pyranisamine maleate (neoantergan), cortisone, or sodium sulfadiazine. Dimercaprol (BAL) has not protected against 1.5 MLD. Massive doses of heparin do not prevent acute toxic death. Thus the mechanism of injury is not through intravascular clotting as it is with the toxic principle associated with the Rous sarcoma (90). Toxotoxin is not hemolytic (103) and preliminary experiments have not revealed antiacetylcholinesterase activity (47a).

The chemical constitution remains to be determined. Activity is reduced below detectable levels by crystalline trypsin so that the compound is a protein or contains a trypsin-digestible protein fraction upon which activity depends. Dialysis indicates that toxicity is not associated with a substance of low molecular weight.

Toxotoxin has shown no marked antigenicity. Infected mice die of the same doses that kill normal mice. Titers are not reduced by *in vitro* incubation with immune sera from rabbits or with human sera with dye-test titers of

[4] Very kindly furnished by Dr. H. N. S. Greene.

1/4000,[5] with or without added complement; likewise there is no protection afforded by these sera *in vivo*. Hemagglutination of chicken red blood cells to titers above 1/10 has not been obtained. Material used in skin tests (26) and in the dye test (85) very probably contains the toxin; whether it plays any important part in these reactions has not been determined.

Animal infections, natural.—Spontaneous infection of a wide range of animals has been reported (115, 109, 11). There is reason to doubt some reports involving birds and heterotherms (see below) and at various times *Encephalitozoon* may have been mistaken for *Toxoplasma*. The following data are considered unquestionable: Toxoplasmosis has appeared in epizoötic form in pigeons (44, 112) and rabbits (112) and occurs enzoötically in wild hares in Denmark (13). In England it is so common in voles (*Microtus agrestis*) as to be credited with being a population-limiting factor (27, 24). Domestic or farm animals, dogs and cats have frequently been found infected (69, 41, 70). It is common in rodents, and 9 per cent of trapped wild rats in Savannah, Georgia (U.S.A.), were toxoplasma-carriers (73).

It is important to emphasize that stock strains of all laboratory animals commonly used for toxoplasma work have been found spontaneously infected: rabbits (48), guinea pigs (66, 62), and mice (66, 61). This fact should always be taken into account in evaluating isolation experiments or the purity of any strain. Although infected immature eggs have been described (91), no record of infection in viable chick embryos has been found.

Experimental animal infections.—No proved case of failure to infect a warm-blooded animal by inoculation is known to the reviewer. Conversely, it has not yet been proved that the organism has more than a short life in cold-blooded vertebrates (56), or that multiplication takes place in arthropoda (7). Since old records of infected birds are open to question, it is well to point out that both the domestic fowl and pigeon are spontaneously infected and experimentally susceptible (44, 112, 91, 56), and that ducks, canaries, English sparrows, song sparrows, and purple grackles (of which the last two are migratory) have been infected with toxoplasmata from mice (56).

Acute fatal infections are more commonly seen in rodents than in large mammals. Monkeys and apes are relatively tolerant; only one of eleven primates, an immature *Macaca mulatta*, died after inoculation with a human strain, and at least ten of these received intracerebral injections (16); furthermore, there are eleven additional reports in the literature of nonfatal infections, including two in baboons (16). Whether man is similarly tolerant will be discussed later.

Although eggs have been suggested for primary isolation, comparative studies on sensitivity have indicated the superiority of mouse brain inoculation, even with the disparity in volumes of inoculum (104). The organism

[5] I am indebted to Drs. R. S. Beckett and H. A. Feldman for these sera.

grows equally well in embryos 6 to 12 days old, with secondary incubation at either 35° or 37°C.; the highest concentrations are found in the embryo, which dies on about the eighth day, and in the chorioallantoic membrane (101). Illustrations of the lesions have been published (101, 64).

Strains.—Variation in virulence according to host of origin has been the difference chiefly noted amongst strains of *Toxoplasma*. Repeated passage usually increases the fatality rate for the species employed, and strains uniformly fatal for mice may be obtained. Strains of low virulence immunize mice against fatal strains even following intracerebral injection (105). Chick embryo passage may (52) or may not (53) decrease the MLD for mice. Slight differences in size and shape according to host have been reported (40). Drug susceptibility probably varies with the strain and also possibly with the capacity to produce pseudocysts (see: *Chemotherapy*).

Toxoplasmosis

The interaction between the microörganism and its environment, man and other vertebrates, is discussed in the remainder of this review. All human data are from cases authenticated by isolation of the organism or by detection of toxoplasma in tissues. Presumptive human cases based on serological data and symptoms are *sub judice*, and information from such cases is not utilized unless so stated.

Immunity.—There is probably no innate immunity to infection in individuals of susceptible species. Acquired immunity to reinfection has been known for a long time [Laveran & Marullaz (47b)]. Potentially fatal strains may produce nonfatal infections in mice if administered in small numbers or by the subcutaneous route, or if appropriate therapy is provided (105, 106). Recovered mice then survive reinoculation with fatal doses of homologous or heterologous strains, even under such severe conditions of testing as the intracerebral inoculation (105) of at least 5000 LD50 by the 10th day (31). Reinoculations result in reinfections (78) but acquired immunity endows the animal with the ability to control the second as well as the first infection. Acquired resistance is correlated with retention of organisms in the tissues, particularly in the brain. The situation when first observed appeared paradoxical, for in immune animals organisms which are ordinarily highly destructive are lodged in particularly susceptible neural tissue without producing death or even conspicuous signs of illness. Pathological studies furnished the clue. The brain contains pseudocysts often in great number, but it shows little or no pathological change in immediate proximity to these pseudocysts or elsewhere, and isolated extracystic organisms are not found (105). Lack of tissue destruction is not due to lack of virulence, for the brain which contains pseudocysts produces fatal infections in new mice (105). Thus, virulent organisms enclosed in pseudocysts may be completely innocuous to susceptible tissue of the mouse, and the same conclusion has been reached from studies of human cases (55, 92).

In these chronic infections with virulent organisms the immunological

balance is delicately adjusted, since defense mechanisms of the host do not lead to elimination of organisms, at least not for long periods of time, yet they adequately prevent marked multiplication, the attendant tissue destruction, and resultant symptomatology. The explanation of this state of affairs offered by the reviewer was that the pseudocyst membrane serves to prevent outward diffusion of destructive substances and perhaps also to retard inward penetration of antibodies and chemotherapeutic agents (105). Subsequent observations have supported this hypothesis. Pseudocysts remain in brain tissue after the acute infection is cured by chemicals known to cross the blood-brain barrier (106), and the pseudocyst membrane considerably retards penetration of even as small a molecule as that of hydrochloric acid (19).

Brain pseudocysts persist for at least two years (80). Chronic *Toxoplasma* infection does not relapse and become acute following splenectomy or X-ray irradiation, even at lethal doses (35). Healthy human carriers do not become ill during or following pregnancy.

Immunology and serology.—Serological reactions have been described in profusion since the original report of complement fixation with the serum of infected animals [Nicolau & Ravelo (68)]. Protection tests in mice (86), quantitative neutralization tests in rabbits (86, 87), pock count methods in eggs (53), cutaneous sensitivity tests in man (33, 26), and the dye test, which merits special attention, have all been investigated. The dye and complement fixation tests appear currently to be preferred. For complement fixation a variety of antigens have been used in an effort to obtain high sensitivity and to avoid anti-complementary activity (102, 81, 108, 8, 91a). It is not known whether results with the various antigens are strictly comparable.

The relation of these antibodies to immunity requires clarification. In the presence of dye antibody titers of 1:4096, organisms continued to be isolated from the blood [Verlinde & Mastenieks (99); Verlinde *et al.* (100)], and a fatal outcome was not prevented although dye-test titers of 1:16,384 were reached at least 28 days before death [Kass *et al.* (45)]. In treated mice dye-test titers were in the same range whether the animals lived or died [Frenkel (31)].

A very original contribution to immunological studies of toxoplasmosis, with possible wider applications, was made by Sabin & Feldman (85) with the dye test. In this test, toxoplasmata in mouse ascitic fluid are exposed to specific antibody, then to a complement-like substance "activator," and finally to methylene blue. In the presence of antibody the cytoplasm remains colorless or becomes only faintly stained although the nucleus stains blue. By diluting the serum and establishing a 50 per cent stain end-point, quantitative results may be obtained. The reaction is correlated with a pH change of the cytoplasm and is believed to be caused by loss of cytoplasmic affinity for this stain.

This test has been very widely used, duplicate end-point titers have been

obtained from independent laboratories, and the test is probably a very sensitive one. Rising titers to high final values (1:250 to 1:4000 or more) are obtained in acute toxoplasmosis, and high titers also occur in mothers of congenitally infected infants. The significance of the high incidence of low-titered sera in the general population will be discussed later. Sera from acute cases may show a low titer at the outset but this undergoes a marked rise two weeks later.

The specific antibody remains active after exposure to 56°C. for 30 min.; activator does not. Animal sera are not used for activator since they may inhibit staining through their content of nonspecific substance which, unlike the specific antibody, is thermolabile. Although this nonspecific thermolabile substance has never been encountered in human sera by persons with great experience with the test (25), one group of workers claims that activator sera must be carefully chosen to avoid this source of confusion (111).

The dye-test mixtures are extremely complex and contain organisms, heparin (which is anticomplementary), the skin test antigen, toxotoxin, the test serum, and complement or activator or both. Which components may be unessential has not been determined. Although various modifications of the original procedure have been introduced, it is probably preferable to follow the original procedure if comparable results are desired. Dr. Feldman has very kindly made available his current technique, which is essentially the same as that first described.[6]

Chemotherapy.—Much labor and scanty yield characterize the results in this field. Toxoplasmosis is only slightly influenced by the majority of compounds active in protozoan and bacterial infections (102, 21, 32, 42). *In vitro* screening is particularly unsatisfactory; notably, the activity of the sulfonamides would not have been predicted from such methods (102). What has become clarified are the conditions of testing and the degrees of effectiveness. An ideal chemotherapeutic agent will do at least two things: (*a*) arrest progress of the infection even when given late in the disease; (*b*), prevent or cure carriers by preventing formation of pseudocysts or by destroying them if already formed. No substance now known will always accomplish either of these two ends, far less both simultaneously.

The effectiveness of known agents varies inversely with the duration of

[6] The dye test is performed as follows: Aspirate peritoneal fluid from mice on the 3d or 4th day following intraperitoneal infection. If toxoplasmata are abundant, dilute exudate 1 part with 5 of defibrinated human blood containing activator and heparin added to 1:5000 concentration. Test sera are inactivated, and tubed in 0.1 ml. amounts in successive fourfold dilutions. An equal amount of activator-toxoplasma mixture is added and the mixture incubated at 37°C. in a water bath for 1 hour. To tubes removed from the water bath are added 0.02 ml. freshly made methylene blue of pH 11. Counts are made on drops removed from the mixture. Activator serum keeps indefinitely when frozen in dry ice; inactivated test sera and dilutions may be stored frozen for long periods. The end-point serum dilution is the one which produces equal numbers of organisms with stained and unstained cytoplasm.

the infection. Therapeutically worthless compounds can be made to show apparently striking activity if given prophylactically or conjointly with infecting organisms, and although biochemical and metabolic information may be so obtained, such experiments convey no meaning as to curative value.

The time-lapse effect has been shown in experiments with sulfapyridine. Untreated mice died 15 days atfer infection, about 50 per cent died when treatment was delayed 10 days, and nearly 100 per cent survived when treatment was delayed 5 days. Significantly, all recovered animals treated after the fifth day harbored virulent organisms in the brain, and those examined histologically revealed the presence of pseudocysts (106).

Presumably, treatment acts in part directly upon the organisms, and prolongs the life of the host until immune mechanisms become effective. Low-dose treatment with sulfadiazine results in high mortality, a high rate of recovery of organisms after treatment, and complete resistance to reinoculation, whereas high doses lead to a low mortality, a low recovery of toxoplasmata, and only partial resistance to reinfection [Biocca & Nobrega (4)]. This suggests that the high doses tended to sterilize the infection before immunity is complete.

Sabin & Warren (88) first reported on the sulfonamides, noting activity both prophylactic and curative; the latter effect was confirmed by Biocca & Pasqualin (6). Weinman & Berne (106) attempted to define the optimal results which could be obtained with prolonged maximal doses of the drug and reported the time-lapse effect, some cures even late in the infection, and failure to sterilize with maximal doses. Sulfone prophylaxis was demonstrated by Mingoja & Biocca (60) and some curative action by Biocca & Nobrega (19) but none of the sulfones used were superior to sulfadiazine in curing the disease. Eradication of the carrier state with sulfones, which has been reported or suggested (17), needs careful study utilizing a strain known to produce pseudocysts in most or all animals and with prolonged high dosage for maximal effect. Diasone is more active than sulphetrone and promin (17, 18). Avlosulfon compares favorably with diasone in acute infections at one-tenth the dosage (18).

The prophylactic action of sodium sulfathizaole and of 4-4′ diamino diphenyl sulfone was inhibited by simultaneously administered *p*-aminobenzoic acid (PAB), and the first compound by folic acid also (95, 94). *p*-Amino-salicylic acid is reported to be therapeutically inactive (32); sulfamylon, not antagonized by *p*-aminobenzoic acid, is also valueless (21).

Probably sulfadiazine is as good as any of the sulfonamides. Yet in human use it has been disappointing (45), possibly because of unavoidable delay in instituting treatment. Likewise, sulfamethylpyrimidine failed to halt the recovery of viable organisms from both blood and spinal fluid (99).

Claims for activity of the antibiotics are more exuberant than demonstrable. Aureomycin has prophylactic activity which is not nullified by PAB (94); curatively it is ineffective (1, 42). Terramycin and streptocin given in delayed treatment did not prolong the life of mice infected with the RH

strain (103); terramycin has been reported on favorably in rabbit infections although it is not stated when treatment commenced (38).

Trials of combined treatments have not been extensive. Antiserum and sulfadiazine combined were more effective than either (22), whereas endochin (SN 13421) with prophylactic action only, did not enhance the curative action of sulfadiazine (37). Data on acquired drug resistance have not been encountered.

Much of the literature concerning chemotherapy is exceedingly difficult to assess. Reports pleasing to the authors thereof are published with no information as to death rates or survival periods in controls, and with no notation of the period at which treatment was begun or of the comparative carrier rates in cured and untreated animals. Evaluation of a test compound can pose problems, since infection with some strains of *Toxoplasma* may be easier to cure than others. Parallel tests with well-known therapeutic agents, e.g., sulfapyridine or sulfadiazine, should give comparative information of value.

Transmission.—The natural means of transmission is unknown except in the case of congenital transmission. This occurrence has been proved repeatedly in man by the recovery of toxoplasmata from stillborn infants (55), from infants vaginally extracted from the uterus before birth (71), from infants delivered by Caesarian section (115), and from aborted uterine contents (54). Human congenital infection has posed problems of the highest biological and practical interest.

The first evidence ever obtained that *Toxoplasma* infection in man, as in animals, could be tolerated as an asymptomatic infection came from studies of these mothers, for they were invariably in perfect health or presented only slight complaints which thus far have not been proved to be the result of the infection itself. The reviewer suggested a decade ago (104) that the facts could be explained if we assumed that in man, as in animals, asymptomatic infection is correlated with chronic infection; that the chronic infection in man would prove to be further correlated with pseudocyst formation, as it is in animals; and that the congenital human cases represent the transmission from a chronic tolerant carrier to the infant.

According to this view, the infant is a nonimmune intruder into an environment where a satisfactory balance has been established between virulent organisms and the immune responses of the mother. Proof requires that three points be established: the existence of asymptomatic infection in man, chronic infection in man with few or no symptoms, and asymptomatic chronic infections in pregnant women followed by birth of an infected, ill infant.

There can be no doubt that asymptomatic infections exist, as witnessed by the symptomless histories of the mothers cited above. Furthermore, following Tomlinson (98), pseudocysts containing toxoplasmata have been repeatedly described in humans who have died of causes unrelated to toxoplasmosis such as suicide, drowning (46, 55), or other diseases (76). These

pseudocysts had provoked no tissue destruction nor reaction, and are pathological evidence of an infection which has progressed beyond the initial stages. Finally, the evidence reported by Wilder (113) of the finding of *Toxoplasma* in eyes of 41 adults suffering from longstanding retinitis of a type known to be caused by toxoplasmosis is the final evidence needed for the existence of chronic infection.

Thus far no single individual has yet been found who fits into the category of being both a chronic carrier and a pregnant female. However, since no marked difference in sex incidence is known, if one admits that chronic human infection occurs, then it is tantamount to certainty that child-bearing females will be found in this category. Evidence of another sort, derived from population surveys either by skin testing or serological techniques, has revealed an incidence of positive tests which suggests widespread infection, increasing with age, in persons with no complaints or illness. This constitutes agreement with the views put forth above (see: *Epidemiology*).

This view has important practical consequences but has not won universal acceptance. The alternative explanation offered is that infection is contracted during pregnancy. This may and probably does happen from time to time, but if it is assumed to be the usual occurrence then we are left with no explanation whatsoever as to why the mothers tolerate the infection so very well. Furthermore, it is difficult to comprehend why pregnant females should be especially susceptible to infection during this particular nine-month period of their lives. Finally, this view is not in accordance with the population surveys cited above. It is a striking oddity that some of the very persons who accept the view that toxoplasmosis is widespread in the population are unwilling to admit that certain segments of that population become gravid.

The concept that most cases of congenital infection develop in a mother who is immune throws light upon still another puzzling feature of toxoplasmosis, namely, the finding that congenitally infected infants have a disease most often limited to the central nervous system, whereas in adults the heart, lungs, skin, and muscles are chiefly involved. There is no anatomical explanation for this; infection which reaches the fetus must pass either through the fetal membranes or across the placenta with the blood (63).[7] If the latter occurs, as seems probable, the fetal liver, heart, and lungs are more exposed to infection than the brain. It is known, however, that antibodies are transmitted quantitatively from mother to infant (84). If we admit then that the fetus receives both organisms and antibodies from the mother, and coexistence of both in the blood stream is known (99, 100), an explanation becomes evident. Antibodies control infection throughout the rest of the body, but do not penetrate in equally effective amounts into the central nervous system of the fetus. If this view is correct, antibody titers in the blood should

[7] Very recently toxoplasmata have been found in the placenta by Christen *et al.* (12a), by Neghme *et al.* (64a), and by R. S. Beckett (1a).

be higher than in the cerebrospinal fluid of infants, and this indeed is the case in the very few reports available (2, 24a, 97). Systematic comparative titrations should show the correctness or falsity of this view. It is not without significance perhaps that when infected newborn mice are treated with sulfadiazine and serum, lesions are almost limited to the brain, whereas with sulfadiazine alone there are scattered lesions throughout the liver, lung, spleen, brain, and kidney (22).

After one toxoplasma-infected child is born, subsequent infants are normal, or at least have not been proved to be infected. The reason for this is not known. Other protozoan infections such as malaria (63) are not invariably transmitted to all offspring, and an analogous situation may exist in toxoplasmosis. Possibly the immune mechanism of the mother may be greatly strengthened by antigens from foci of proliferating microörganisms in the fetus which diffuse across the placenta. This is not proved, but the mothers usually show very high titers of antibodies, very much higher than those of the general population and at a level comparable to that observed in acute infections.

Other avenues of infection must exist but their importance in natural transmission has not been established. Transmission via the milk to nursling mice occurs (23). *Toxoplasma* may be infective when fed (59, 67, 27), but this is not invariably the case (1). In animals, toxoplasmata have been found at the surface of the stomach and intestine and in the kidneys; thus a possible transmission cycle exists following ingestion of feces and urine. The role of arthropoda has been repeatedly suggested (12, 74), but neither transmission under conditions resembling natural circumstances nor even multiplication in insects has been demonstrated (7). Limited survival of organisms occurs in a variety of insects but it remains to be shown that this is significant. There is one report of infection following inoculation of "wild" ticks—toxoplasmata were noted after animal passages, but there is no proof that the infection originated in the ticks (39).

Epidemiology.—Epizoötics with high mortality occur in rabbits and pigeons confined to close quarters (44, 112). Infection exists enzoötically in wild animals, such as hares (13), in semidomestic animals such as rats, and has repeatedly been reported in domestic varieties, e.g., dogs and cats (41, 69). High incidence rates were found for rats, with 14 of 160 trapped specimens infected (73). Higher death rates from spontaneous infections have been noted several times in the winter season (12, 13).

Prevalence of human infection.—The incidence of infection in man is a major unsolved problem. In addition to persons acutely ill with toxoplasmosis there are numbers who are infected but show no evidence of this. Although isolation of toxoplasmata from such persons is usually not accomplished, yet there is every reason to suppose that the proved cases constitute only a portion of the infected total (see: *Transmission*). Various immunological tests have therefore been applied to the problem. The most recent reports are startling. Both the dye test (25) and the skin test (26) have been used

with consistent results. The number of positives in the population varies with the age group. It is low in the first decade of life, then increases to reach a maximum after 40 years of 30 per cent (33) and even 60 per cent (25, 26) of the normal population. These figures vary in different reports, but there is essential agreement that an important segment of the adult population give positive reactions in tests which so far are not known to be falsely positive.[8]

Since the dye and complement fixation tests can be quantitated and the skin test cannot, population surveys using these methods may prove of particular value. A dye-test titer as low as 1:40 was reported in a woman later proved to be a carrier [Magnusson (54)]. Feldman (25) adopted a 1:16 titer as significant in population surveys, and in Pittsburgh, Pennsylvania, found the incidence of dye-test titers at this level or higher to be: 5 per cent at 0–4 years, 10 per cent at 5–9 years, 25–30 per cent at 10–29 years, 45 per cent at 30–39 years, 65–70 per cent at 40–60 years and above. Data on the distribution of antibody levels in the general population are scanty; one report states that 10 per cent of 640 sera titered above 1:100 in the dye test [Verlinde & Makstenieks (99)].

At present, the relation of positive skin and low titer serological tests to *Toxoplasma* infection is conjectural. If these are taken at face value to signify infection, then toxoplasmosis is one of the commonest infections of man —a conclusion which cannot justifiably be drawn on present evidence. It is beside the point to emphasize that the various tests are in agreement; this may only indicate that the argument is circular and has no inconsistencies. The matter can be removed from the realm of opinion by an approach permitting some means of correlating serological reactions with actual infections. It would seem that such an approach is possible and would be highly informative.

LITERATURE CITED

1. Adams, F. H., and Cooney, M., *Proc. Soc. Exptl. Biol. Med.*, **70**, 258–60 (1949)
1a. Beckett, R. S. (Unpublished data)
2. Binkhorst, C. D., *Toxoplasmosis. A Clinical, Serological and Histopathological Study with Special Reference to the Eye Manifestations* (Stenfert Kroese, Leiden, Holland, 163 pp., 1948)
3. Biocca, E., *Riv. Parassitol.*, **10**, 73–92 (1949)
4. Biocca, E., and Nobrega, P., *Rend. accad. nazl. Lincei, classe sci. fis., mat. e nat.*, **2**, 353–59 (1947)
5. Biocca, E., and Nobrega, P., *Arquiv. biol. (São Paulo)*, **30**, 63–66 (1946)

[8] These figures vary according to locality. Gard (35a) reported positive skin tests in 46 per cent of 1894 persons in Stockholm and in only 10 per cent of adults in northern Sweden. Frenkel (33) reported 28 per cent positive skin tests in an age group over 50, while Westphal in Hamburg (110) found 18.6 per cent "significant dye tests" in suspected patients and parents, and only 2 per cent in other adults. Mühlpfordt (63a) states that animals inoculated with or fed *Sarcocystis* subsequently react positively in the *toxoplasma* dye test. However, human infection with *Sarcocystis* is rare.

6. Biocca, E., and Pasqualin, R., *Ann. igiene*, **52**, 12–14 (1942)
7. Blanc, G., Bruneau, J., and Chabaud, A., *Ann. inst. Pasteur*, **78**, 277–280 (1950)
8. Bozicevich, J., in *Parasitic Infections in Man*, Chap. 4, 37–55 (Most, H., Ed., Columbia University Press, New York, N. Y., 1951)
9. Brug, S. L., and Vos, J. J. T., *Nederland. Tijdschr. Geneesk.*, **86**, 2956–63 (1942)
10. Brumpt, E., *Précis de Parasitologie* (Masson & Cie., Paris, France, 2138 pp., 1949)
11. Callahan, W. P., Jr., Russell, W. O., and Smith, M. G., *Medicine*, **25**, 343–97 (1946)
12. Chatton, E., and Blanc, G., *Arch. inst. Pasteur Tunis*, **10**, 1–40 (1917)
12a. Christen, R., Agosín, M., Pino, F., Jarpa, A., Thiermann, E., and Neghme, A., *Rev. med. Chile*, **79**, 714–16 (1951)
13. Christiansen, M., and Siim, J. C., *Lancet*, **I**, 1201–03 (1951)
14. Cowen, D., and Wolf, A., *J. Neuropathol. Exptl. Neurol.*, **10**, 142–57 (1951)
15. Cowen, D., and Wolf, A., *J. Exptl. Med.*, **92**, 417–29 (1950)
16. Cowen, D., and Wolf, A., *J. Infectious Diseases*, **77**, 144–57 (1945)
17. Cross, J. B., *Proc. Soc. Exptl. Biol. Med.*, **76**, 548–51 (1951)
18. Cross, J. B. (Personal communication)
19. Cross, J. B., *J. Infectious Diseases*, **80**, 278–96 (1947)
20. Cross, J. B., and Anigstein, L., *Am. J. Trop. Med.*, **29**, 473–81 (1949)
21. Cross, J. B., and Anigstein, L., *Texas Repts. Biol. Med.*, **6**, 260–65 (1948)
22. Eichenwald, H., *Proc. Soc. Exptl. Biol. Med.*, **71**, 45–49 (1949)
23. Eichenwald, H., *Am. J. Diseases Children*, **76**, 307–15 (1948)
24. Elton, C., Davis, D. H. S., and Findlay, G. M., *J. Animal Ecol.*, **4**, 277–88 (1935)
24a. Farquhar, H. G., and Turner, W. M. L., *Arch. Disease Childhood*, **24**, 137–42 (1949)
25. Feldman, H. A. (Personal communication)
26. Feldman, H. A., and Sabin, A. B., *Pediatrics*, **4**, 798–804 (1949)
27. Findlay, G. M., and Middleton, A. D., *J. Animal Ecol.*, **3**, 150–60 (1934)
28. França, C., *Jornadas sci. mat. fis. nat. acad. sci. Lisboa*, **1**, 221–29 (1917)
29. França, C., *Jornadas sci. mat. fis. nat. acad. sci. Lisboa*, **1**, 26–65 (1917)
30. Frenkel, J. K. (Personal communication)
31. Frenkel, J. K., *Federation Proc.*, **11**, 468–69 (1952)
32. Frenkel, J. K., *J. Am. Med. Assoc.*, **140**, 369–77 (1949)
33. Frenkel, J. K., *Am. J. Ophthalmology*, **32**, 127–35 (1949)
34. Frenkel, J. K., *Proc. Soc. Exptl. Biol. Med.*, **68**, 634–39 (1948)
35. Frenkel, J. K., Jacobs, L., and Melton, M. L., *Am. J. Path.* (In press)
35a. Gard, S., *Nord. Med.*, **45**, 352–57 (1951)
36. Gard, S., Magnusson, J. H., Wahlgren, F., and Gille, G., *Pediatrics*, **4**, 432–42 (1949)
37. Gingrich, W. D., and Darrow, E. M., *Am. J. Trop. Med.*, **31**, 12–17 (1951)
38. Giroud, P., and Gaillard, J. A., *Compt. rend.*, **232**, 1457–59 (1951)
39. Giroud, P., and Grjebine, A., *Bull. soc. path. exotique*, **44**, 54–57 (1951)
40. Guimarães, N. F., *Mem. inst. Oswaldo Cruz.*, **38**, 257–320 (1943)
41. Hagan, W. A., and Bruner, D. W., *The Infectious Diseases of Domestic Animals* (Comstock Publishing Co., Inc., Ithaca, N. Y., 920 pp., 1951)
42. Hogan, M. J., *Ocular Toxoplasmosis* (Columbia University Press, New York, N. Y., 86 pp., 1951)
43. Jacobs, L., and Jones, F. E., *J. Infectious Diseases*, **87**, 78–89 (1950)

44. Johnson, C. M., *Ann. Rept. Gorgas Mem. Lab.*, **1943**
45. Kass, E. H., Andrus, S. B., Adams, R. D., Turner, F. C., and Feldman, H. A., *Arch. Internal Med.*, **89**, 759–82 (1952)
46. Kean, B. H., and Grocott, R. G., *Am. J. Trop. Med.*, **27**, 745–48 (1947)
47. Kean, B. H., and Grocott, R. G., *Am. J. Path.*, **21**, 467–83 (1945)
47a. Kisliuk, R. (Personal communication)
47b. Laveran, A., and Marullaz, M., *Bull. soc. path. exotique*, **6**, 460–68 (1913)
48. Levaditi, C., Sanchis-Bayarri, V., Lépine, P., and Schoen, R., *Ann. inst. Pasteur*, **43**, 673–736 (1929)
49. Levaditi, C., Sanchis-Bayarri, V., Lépine, P., and Schoen, R., *Ann. inst. Pasteur*, **43**, 1063–80 (1929)
49a. Lillie, R. D., *Stain Technol.*, **26**, 123–36 (1951)
50. Lillie, R. D., *Histopathologic Technic* (The Blakiston Co., Philadelphia, Pa., 300 pp., 1948)
51. Lillie, R. D., *J. Lab. Clin. Med.*, **32**, 76–88 (1947)
52. Macdonald, A., *Lancet*, **I**, 950–53 (1949)
53. MacFarlane, J. O., and Ruchman, O., *Proc. Soc. Exptl. Biol. Med.*, **67**, 1–4 (1948)
54. Magnusson, J. H., *Nord. Med.*, **45**, 344–49 (1951)
55. Mantz, F. A., Jr., Dailey, H. R., and Grocott, R. G., *Am. J. Trop. Med.*, **29**, 895–908 (1949)
56. Manwell, R. D., Coulston, F., Binckley, E. C., and Jones, V. P., *J. Infectious Diseases*, **76**, 1–14 (1945)
57. Manwell, R. D., and Drobeck, H. P., *Exptl. Parasitol.*, **1**, 83–93 (1951)
58. Mesnil, F., *Bull. inst. Pasteur*, **16**, 71–73 (1918)
59. Mesnil, F., and Sarrailhé, A., *Compt. rend. soc. biol.*, **74**, 1325–27 (1913)
60. Mingoja, Q., and Biocca, E., *Arquiv. biol.* (*São Paulo*), **27**, 2–8 (1943)
61. Mooser, H., *Schweiz. med. wochschr.*, **80**, 1399–1400 (1950)
62. Mooser, H., *J. Infectious Diseases*, **44**, 186–93 (1929)
63. Morison, J. E., *Foetal and Neonatal Pathology* (Butterworth & Co., Ltd., London, England, 366 pp., 1952)
63a. Mühlpfordt, H., *Z. Tropenmed. Parasitol.*, **3**, 205–15 (1951); *Trop. Diseases Bull.*, **49**, 322 (1952)
64. Mulé, F., *Pediatria*, **48**, 380–92 (1950)
64a. Neghme, A., Thiermann, E., Pino, F., Christen, R., and Agosín, M., *Bol. inform. parasit. Chile*, **7**, 6–8 (1952)
65. Nicolau, S., *Bull. inst. Pasteur*, **38**, 238 (1940)
66. Nicolau, S., and Balmus, G., *Compt. rend. soc. biol.*, **115**, 959–62 (1934)
67. Nicolau, S., and Balmus, G., *Compt. rend. soc. biol.*, **113**, 1002–05 (1933)
68. Nicolau, S., and Ravelo, A., *Bull. soc. path. exotique*, **30**, 855–59 (1937)
69. Olafson, P., and Monlux, W. S., *Cornell Vet.*, **32**, 176–90 (1942)
70. Otten, E., Westphal, A., and Kajahn, E., *Klin. Wochschr.*, **29**, 343–46 (1951)
71. Paige, B. H., Cowen, D., and Wolf, A., *Am. J. Diseases Children*, **63**, 474–514 (1942)
72. Perrin, T. L., *Arch. Path.*, **36**, 568–78 (1943)
73. Perrin, T. L., Brigham, G. D., and Pickens, E. G., *J. Infectious Diseases*, **72**, 91–96 (1943)
74. Pinkerton, H., and Henderson, R. G., *J. Am. Med. Assoc.*, **116**, 807–14 (1941)
75. Pinkerton, H., and Weinman, D., *Arch. Path.*, **30**, 374–92 (1940)
76. Plaut, A., *Am. J. Path.*, **22**, 427–31 (1946)

77. Ray, H. N., and Raghavachari, K., *Indian J. Vet. Sci.*, **11**, 28–32 (1941)
78. Rodhain, J., *Compt. rend. soc. biol.*, **144**, 719–22 (1950)
79. Rodhain, J., *Compt. rend. soc. biol.*, **144**, 717–19 (1950)
80. Ruchman, I., and Fowler, J. C., *Proc. Soc. Exptl. Biol. Med.*, **76**, 793–96 (1951)
81. Sabin, A. B., *Pediatrics*, **4**, 443–53 (1949)
82. Sabin, A. B., *Advances in Pediatrics* (Interscience Press, New York, N. Y., 1942)
83. Sabin, A. B., *Proc. Soc. Exptl. Biol. Med.*, **41**, 75–80 (1939)
84. Sabin, A. B., and Feldman, H. A., *Pediatrics*, **4**, 660–64 (1949)
85. Sabin, A. B., and Feldman, H. A., *Science*, **108**, 660–63 (1948)
86. Sabin, A. B., and Olitsky, P. K., *Science*, **85**, 336–38 (1937)
87. Sabin, A. B., and Ruchman, I., *Proc. Soc. Exptl. Biol. Med.*, **51**, 1–6 (1942)
88. Sabin, A. B., and Warren, J., *Proc. Soc. Exptl. Biol. Med.*, **51**, 19–23 (1942)
89. Sangiorgi, G., *Resenha Clínico-Cientifica* (São Paulo), **18**, 459–62 (1949); *Excerpta Medica*, **3**, 1196 (1950)
90. Shrigley, E. W., *Ann. Rev. Microbiol.*, **5**, 241–64 (1951)
91. Sparapani, G., *Pediatria*, **58**, 411–14 (1950)
91a. Steen, E., and Kåss, E., *Acta Path. Microbiol. Scand.*, **28**, 36–39 (1951)
92. Steiner, G., and Kaump, D. H., *J. Neuropathol. Exptl. Neurol.*, **3**, 36–48 (1944)
93. Sulkin, S. E., Lodowski, C. H., and Hartman, L. W., *Texas Repts. Biol. Med.*, **8**, 45–51 (1950)
94. Summers, W. A., *Am. J. Trop. Med.*, **29**, 889–93 (1949)
95. Summers, W. A., *Proc. Soc. Exptl. Biol. Med.*, **66**, 509–11 (1947)
96. Thiel, van, P. H., *Documenta Neerlandica Indonesica Morbis Tropicis* (Amsterdam), **1**, 264–69 (1949)
97. Thiermann, E., *Bol. inform. parasitarias Chilenas*, **6**, 54–55 (1951)
98. Tomlinson, W. J., *Am. J. Clin. Path.*, **15**, 123–27 (1945)
99. Verlinde, J. D., and Makstenieks, O., *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **16**, 366–72 (1950)
100. Verlinde, J. D., Makstenieks, O., Beekman, H., and Copper, A. C., *Nederland. Tijdschr. Geneesk.*, **94**, 2746–54 (1950)
101. Warren, J., and Russ, S. B., *Proc. Soc. Exptl. Biol. Med.*, **67**, 85–89 (1948)
102. Warren, J., and Sabin, A. B., *Proc. Soc. Exptl. Biol. Med.*, **51**, 11–14 (1942)
103. Weinman, D. (Unpublished data)
104. Weinman, D., *Puerto Rico J. Pub. Health Trop. Med.*, **20**, 125–61 (1944)
105. Weinman, D., *J. Infectious Diseases*, **73**, 85–92 (1943)
106. Weinman, D., and Berne, R., *J. Am. Med. Assoc.*, **124**, 6–8 (1944)
107. Weinman, D., and Klatchko, H. J., *Yale J. Biol. Med.*, **22**, 323–26 (1950)
108. Weinman, D., and McAllister, J., *Am. J. Hyg.*, **45**, 102–21 (1947)
109. Wenyon, C. M., *Protozoölogy* (William Wood & Co., New York, N. Y., 1563 pp., 1926)
110. Westphal, A., *Z. Tropenmed. Parasitol.*, **1**, 526–32 (1950)
111. Westphal, A., and Mühlpfordt, H., *Z. Hyg. Infektionskrankh.*, **131**, 423–34 (1950)
112. Wiktor, T. J., *Ann. soc. belge méd. trop.*, **30**, 97–107 (1950)
113. Wilder, H. C., *Arch Ophthalmol.*, **47**, 425 (1952)
114. Wolf, A., Cowen, D., and Paige, B. H., *J. Exptl. Med.*, **71**, 187–214 (1940)
115. Wolf, A., Cowen, D., and Paige, B. H., *Am. J. Path.*, **15**, 657–94 (1939)
116. Wolfson, F., *Am. J. Trop. Med.*, **21**, 653–58 (1941)

METABOLISM OF SPIROCHETES[1,2]

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Introduction

Spirochetes represent a kind of no man's land between the true bacteria and protozoa. In the latest edition of *Bergey's Manual of Determinative Bacteriology* (3), one finds the spirochetes classified at the top of the phylogenetic tree of bacteria, suggesting that these organisms more nearly approach the nature of protozoa, the next group of organisms above the bacteria in the evolution of living things. In Wenyon's *Protozoology* (78), spirochetes are included in a separate section and with a separate bibliography, indicating that they have an affinity for the protozoa. This intermediate biologic position and extensive difficulties in growing pathogenic spirochetes *in vitro* have led to neglect in their study. The great diversity of these organisms, their classification, their characteristics, the diseases they cause, and a brief discussion of their treatment have been reviewed previously by Eagle (16) and by Davis (9).

The great medical and public health importance of the diseases produced by pathogenic spirochetes is not within the scope of this paper. However, the high priority given to laboratory and field studies of spirochetal diseases by the World Health Organization is ample recognition of the world-wide medical and public health importance of these infections. Spirochetal diseases and their therapy have been studied extensively by clinicians, veterinarians, pathologists, and pharmacologists. On the other hand, what do we know about the spirochetes themselves and the way they produce disease? What do we know about their chemical composition, their metabolism, and the specific way certain drugs and antibiotics affect them? Actually, our knowledge of these pathogens is extremely meager. With the advent of highly potent antibiotics, we now have weapons to cure and eradicate this group of diseases before we know much about the parasites which cause them.

Pathogenic spirochetes belonging to three distinct genera, *Borrelia*, *Leptospira*, and *Treponema*, include parasites that live primarily in the blood, in the blood and tissues, and in the tissues of the host. A fourth pathogen, *Spirillum minus* (3), of questionable spirochetal affinity, causes a dis-

[1] The survey of the literature pertaining to this review was concluded in November, 1951.

[2] The material reviewed in this paper was compiled in connection with a spirochete research project supported by a grant-in-aid to Harvard University from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

ease known as rat-bite fever and exists primarily in the blood of the infected animal. Although the tissue affinity of the pathogenic spirochetes is the basis for the type of primary disease produced, the parasites can also exist in other parts of the host's body during periods of latency. Nevertheless, their primary tissue affinity and the resultant pathology give us some clues to environmental requirements and possible metabolism of the etiological agents. On the other hand, the clinical infections of man and animals known as relapsing fever, treponematosis (syphilis, yaws, and pinta), and leptospirosis are so drastically different that one might suspect that there is little in common metabolically among the spirochetes causing these clinical infections.

During the primary clinical attack of relapsing fever, a variety of different species of *Borrelia* parasitize the plasma of the blood (9). The recorded accounts suggest that certain species of *Borrelia* multiply slowly in the host with only rare organisms being demonstrable, while with other species the organisms can occur in numbers greater than the circulating erythrocytes, indicating that there is no relationship between the degree of parasitemia and the acute febrile reaction. The biochemical complexity of blood gives us no specific clue as to the nutritional requirements and the metabolism of this type of parasite, other than the requirements for high oxygen tension and a complex substrate needed for survival and multiplication.

In leptospirosis, the initial occurrence of the causative agents, *Leptospira* species, in the circulating blood during the first week or ten days of the febrile illness (4) suggests that these parasites find their way into the blood stream and adapt themselves to the host, as evidenced by growth and multiplication. But their subsequent disappearance from the blood stream in seven to fourteen days and their affinity for the liver and kidneys give us some clues as to the environmental requirements for the survival of these parasites in the host. This invasiveness for the viscera might be the result of the elaboration of antibodies by the host, or it might be that the required nutrients for growth are exhausted from the blood stream during the progression of the primary clinical disease. In acute Weil's disease, caused by *Leptospira icterohaemorrhagiae*, the muscle, kidney, and liver lesions are evidence of the pathogenesis of these spirochetes caused by the secretion of toxic substances or by the competition for and utilization of metabolites needed by the host cells.

Three species of *Treponema*, the causative agents of syphilis, yaws, and pinta, are primarily tissue parasites, being carried passively from one part of the body to another by the blood and lymph, but not multiplying to any demonstrable extent within the blood stream. This tissue affinity suggests immediately that these parasites require an environment with lower oxygen tensions than the parasites causing relapsing fever and leptospiral infections. The variable speed of development of lesions at different stages of the disease in man and susceptible animals also demonstrates that these parasites have tissue requirements best provided in precise, localized areas, such as

the skin, viscera, and the central nervous system. Although this tissue affinity narrows the possible environmental requirements somewhat, our knowledge of the differential chemical and physical composition of these preferred foci again is so meager that we have not been able to duplicate *in vitro* the specific requirements needed for the growth and multiplication of the spirochetes. Gradually, information is accumulating about the chemical composition and biochemistry of blood plasma, serum, and erythrocytes (36, 37, 34), and of the viscera (53) that ought to be of great value in the study of pathogenic spirochetes and other pathogens with organ specificity.

The limited success with *in vitro* cultivation of pathogenic spirochetes belonging to the genera *Borrelia* and *Treponema* has been conditioned by our ignorance of the chemical composition, metabolism, and nutrition of these parasites. The failure to obtain this information has been determined by our inability to obtain sufficient quantities of the organisms for the required study. Some investigators have turned to nonpathogenic spirochetes in an effort to get information that might be applicable to studies of the pathogens. Although this is not an ideal procedure, we can hopefully expect this experimental approach to aid in pointing the way to successful *in vitro* cultivation of the pathogens.

In searching for possible clues to the metabolism of pathogenic spirochetes, available leads are helpful, but we need more biochemical information about the course of infection in the host. In primary relapsing fever, the characteristic febrile reaction, anemia, and splenomegaly without other gross lesions suggest that the parasites excrete certain unknown toxic substances into the blood, perhaps nitrogenous metabolic products, that lead to the characteristic symptomatology of this disease. The varied parasitemia with occasional phenomenal multiplication of these parasites in the blood stream during the primary illness and the rapid resolution of this bloodstream infection by crisis to bring an end to the febrile reaction represent metabolic activity or immunological activity on the part of the host to limit the duration of this infection. The reappearance of antigenically different parasites in the blood stream with subsequent relapse (64) suggests that the parasites have adapted themselves to the resistance of the host and can grow and multiply once again. The ability of these parasites to invade the spleen and the brain and to persist as a latent infection for extended periods of time and their ability to parasitize lice and ticks, the vectors of relapsing fever, suggest that species of *Borrelia* can adapt themselves to extremely different environmental conditions and survive. The infection known as *mal de pinta*, caused by *Treponema carateum*, occurs in dark-skinned races primarily, indicating that this spirochete finds the physiological environment in the skin suitable for its maximum metabolic activity. This localization in the skin and the three distinct types of lesions lead one to believe that the physical and chemical requirements of *T. carateum* differ from those of other species of *Treponema*. Although syphilis and yaws have different clinical characteristics, different methods of transmission, and different response to chemo-

therapeutic agents, they might be considered chronic infections that require considerable time to produce devastating lesions in the host. The existence of primary, secondary, and tertiary phases of the two diseases represents the ability of the parasites to exist not only in widely different environmental conditions, but also under varying patterns of metabolism at different stages of the infection. The phenomena that make up the host defenses to the parasites in certain tissue environments lead, on the one hand, to acute extensive involvement of tissue and death of the host and, on the other hand, to latency or spontaneous cures. The experiences and calculations of Magnuson *et al.* (41), indicating that the Nichols strain of *T. pallidum* divides once in about thirty hours, help to explain the slow development of lesions and increase of spirochetes. The prolonged chronic course and slowly developing immunity of syphilis and yaws are in contrast to the shorter duration and rapid immune response in leptospirosis and relapsing fever.

The studies of Scott and collaborators (65 to 68) on the role of the ground substance and spreading factors or testicular hyaluronidase on the incubation period of experimental syphilis in rabbits and on the histopathological reaction provide a number of leads for future *in vivo* and *in vitro* studies. These investigators were able to demonstrate a shortening of the incubation period by the introduction of testicular hyaluronidase with the inoculum of *T. pallidum* and the prevention of metachromasia in syphilitic lesions by hyaluronidase, suggesting that the characteristic disease production in the testes "is referable to the production of hyaluronic acid and/or a related mucopolysaccharide, possibly chondroitin sulfate." Two possible explanations for the results presented themselves: either hyaluronidase is a growth-promoting substance for *T. pallidum* or this enzyme through its action on the ground substance provides an environment favorable for the growth and multiplication of the spirochete. The evidence to these investigators appeared to favor the second interpretation because of the possibility that mucopolysaccharides in the testicle are peculiarly suited to the growth of *T. pallidum* or that hyaluronidase initiates hydrolysis of complex mucopolysaccharides which can then be continued by an enzyme of the spirochete.

The demonstration by Turner & Hollander (73) that cortisone therapy (1.5 to 3.0 mg. per kg., twice daily) alters the character of lesions in experimental syphilis of rabbits is perhaps directly related to the results of Scott and collaborators (65 to 68). After cortisone treatment, the change of the syphilitic lesions to a soft and spongy consistency with excessive accumulation of a mucoid material, tentatively identified as hyaluronic acid, must hold the explanation for the factors favoring the more rapid growth and multiplication of *T. pallidum* in the lesions.

Thus, the distinct differences in the diseases produced in man by pathogenic spirochetes, the widely differing foci of infection in the host, the varying characteristics of the lesions produced, and the differential susceptibility of the spirochetes to chemotherapeutic agents strongly indicate different types of growth, nutrition, and metabolism. When the needed biochemical

information becomes available, the metabolic pattern among the pathogenic and saprophytic spirochetes will perhaps differ not only from species to species, but also the pattern will differ, in certain subtle ways, from stage to stage of the diseases produced. Unfortunately, the information about metabolism of spirochetes at this time is too scanty to do more than point the way to additional studies that must be done if we wish to understand the nature of these organisms, to explain the way they produce disease, and to understand the mode of life of saprophytic species. No comprehensive coverage of the literature has been attempted because of the lack of quantitative data that are needed before we can explain the metabolism of spirochetes and make comparisons with the better-known bacteria (54, 79), protozoa (46, 40), and animal tissue cells (80, 22).

Chemical Composition

Little is known about the chemical composition of pathogenic spirochetes because of difficulties in obtaining quantities of the organisms in pure suspensions. Methods are now available, however, for obtaining quantities of several species of *Borrelia* from experimental infections, *Leptospira* from cultures, and nonpathogenic species of *Treponema* from cultures; but no *in vivo* or *in vitro* methods to obtain sufficient quantities of the treponemes of syphilis, yaws, or pinta for this type of study are available at present.

Spirochetes of relapsing fever (*Borrelia novyi*) from experimental infections in rats were concentrated by flotation and centrifugation and analyzed for phosphorus components and total nitrogen by McKee & Geiman (44). The initial study of partitioned phosphorus in this parasite was done because high inorganic phosphate favors prolonged survival of this organism *in vitro* and because chemical analysis showed a high amount of alcohol-ether extractable material comprising up to 45 per cent of total dry weight. The method of Schmidt & Thannhauser (63) was used to get the following values for 1×10^{12} organisms: lipid phosphorus, 2.01 mg.; lipid; 382 mg.; cholesterol, 73 mg.; desoxyribonucleic acid phosphorus, 0.694 mg.; ribonucleic acid phosphorus, 1.565 mg.; phospho-protein phosphorus, 0.606 mg.; inorganic phosphorus, 0.382 mg.; 15-min. hydrolyzable phosphorus, 0.691 mg.; and residual organic phosphorus, 0.382 mg. The total nitrogen of a concentrate of 1×10^{12} isolated spirochetes was 49.5 mg. (45) with approximately 65 per cent protein nitrogen, 10 per cent lipid nitrogen, and 25 per cent non-protein nitrogen. Qualitative paper chromatograms indicated relatively large amounts of aspartic and glutamic acids, with smaller amounts of serine, cystine, glycine, threonine, alanine, valine, tryptophane, leucine, isoleucine, phenylalanine, and proline. The quantitative studies on amino acid analysis are not yet completed. Gelperin (26), in an analysis of Reiter spirochetes produced in culture, obtained the value of 58.5 μg. nitrogen for 1×10^{9} organisms that had a dry weight of 0.8 mg. No values for the total nitrogen and amino nitrogen for other species of spirochetes were found. Marshall (43) determined the dry protein content of concentrated *L. icterohaemorrhagiae*

from culture and obtained a range of 1.5 to 2.5 mg. per ml. (using approximately 1×10^6 organisms).

The techniques of histochemistry and cytochemistry (10, 27) to provide information about the composition of spirochetes have been inadequately explored. In general, the pathogenic spirochetes are gram negative; and, except for the stainability of species of *Borrelia* with aniline dyes, *Treponema* and *Leptospira* species are stained with great difficulty when routine dyes are used. The staining procedure used by Robinow (56) to differentiate deeply-staining elements in bacterial cells is unsuccessful with *Borrelia* and *Treponema*. These organisms are homogenous in their normal microscopic appearance within the cellular membrane, but granules of unknown composition and significance have been detected in their cytoplasm with the aid of the electron microscope (39, 47). Van Thiel & Van Iterson (75) failed to detect any cytoplasmic granules in *Leptospira biflexa* with the electron microscope.

Respiration and Carbohydrate Metabolism

The earliest reference found to studies on the respiration of *Borrelia* species was that of Fenyvessy & Scheff (21). These authors compared the respiration of relapsing fever spirochetes from experimental infections in white rats with that of trypanosomes. The development of our knowledge of relapsing fever and human trypanosomiasis has made the above study a fortuitous and an interesting one because of similarities in the relapsing nature of these two unrelated infections, in their immunology, and in their susceptibility to certain heavy metals used as therapeutic agents.

Using the Warburg technique with a water-bath temperature of 26°C., Fenyvessy & Scheff (21) found that relapsing fever spirochetes do not utilize oxygen in contrast to high oxygen consumption by trypanosomes. Glucose utilization by the spirochetes was demonstrated to be glycolytic, the amount being dependent on the number of spirochetes present (no relative amounts were given). Scheff (62) continued and extended these studies to carbohydrate metabolism of the spirochetes of relapsing fever (*B. recurrentis*) and of fowl spirochetosis (*Spirochaeta gallinarum* = *B. anserina*). These extended studies further confirmed the fact that oxygen was not utilized and that carbon dioxide was formed in considerable amounts. Scheff (62) even stated that these organisms must be classified as true anaerobes. Glucose was essential in the medium to maintain motility and viability of the spirochetes, but the amount of lactic acid formed could not be accounted for entirely by the amount of glucose utilized. In more recent *in vitro* studies at 37°C. with suspensions containing 1×10^{12} *B. novyi*, McKee & Geiman (44) confirmed the use of large quantities of glucose (0.4 millimole/hr.). Only 65 per cent of the glucose utilized was accounted for as accumulated lactic acid. By calculation, approximately 10 per cent of the glucose utilized could have been oxidized completely to carbon dioxide and water. The detection of a low-grade oxygen consumption is significant, but here again these stud-

ies need to be elaborated upon in an effort to explain the localization of these parasites in the blood with its high oxygen tension.

Obviously, oxygen is not toxic to *B. novyi* as it is to *T. pallidum*, a spirochete that requires an anaerobic environment for survival and propagation. *In vitro* studies with *T. pallidum* have demonstrated that maximum survival of the parasite of syphilis is dependent on an anaerobic gas phase produced by reducing substrates, by sealing inoculated media (51), or by the maintenance of anaerobiosis by passing a mixture of 5 per cent carbon dioxide and 95 per cent nitrogen through the culture vessels (48). The fundamental respiratory processes of *T. pallidum* can be studied only when this parasite can be produced in quantities needed for studies by the Warburg technique or until the Cartesian diver technique (27) is explored and adapted for studies on respiration of concentrated suspensions now available from experimental infections in rabbits.

In a series of studies on *L. icterohaemorrhagiae* in cultures, Chang (8) suggested that the nature of respiration of this species of *Leptospira* may be quite different from that of common bacteria since no simple sugars, such as glucose, galactose, mannitol, maltose, and fructose are utilized. Although this parasite survives as long as 22 days at environmental temperatures in water leading to transmission of the infection (74); and, although maximum growth of the organisms in culture requires oxygen, Chang (8) failed to detect any consumption of oxygen by 18 million *Leptospira* in 6 hr. using the Warburg technique. Marshall (43) succeeded however in measuring the oxygen consumption of *L. icterohaemorrhagiae* by using greater concentrations of spirochetes. The organisms were grown at 28°C. for seven to ten days in Korthof's medium and concentrated suspensions were obtained by centrifuging 250 to 500 ml. of culture after addition of saponin to a final concentration of 1:1000. Organisms from heavy cultures showed a slow oxygen uptake over a period of 4 hr. ranging from 9.71 to 49.4 μl. The oxygen uptake of concentrated suspensions (6×culture concentration of 10^8/ml.) reached 151 μl. in 4 hr. Again, no stimulation of respiration was produced by adding lactose, maltose, mannitol, galactose, DL-methionine, L-tryptophan, or L-tyrosine and the amino acids actually depressed respiration. No utilization of glucose by *Leptospira* growing in Korthof's medium could be demonstrated. Respiration was stimulated by the addition of rabbit serum, but not by Witte's peptone or haemoglobin. Marshall (43), as a result of these experiments, pointed out that stimulation of respiration in short-term experiments does not rule out the necessity of amino acids and possible heme compounds for growth and reproduction. Glucose, however, is not the source of energy for *Leptospira*, but rabbit serum apparently contains an important thermolabile energy-providing substrate or a vital cofactor. The protein-binding action of serum albumin in removing substances toxic for respiratory activity and the testing of plasma fractions for stimulating respiratory activity of *Leptospira* need to be explored in attempts to identify those substances needed for metabolism and growth. Chang (8), in analyzing his re-

sults with *L. icterohaemorrhagiae*, expressed the belief that ". . . the leptospira lives chiefly, if not exclusively on proteins." This statement needs to be qualified, however, and studied by additional experiments, because this investigator presented evidence elsewhere in the same paper indicating that vitamins and glycerol (a possible energy source) appeared to be essential for growth. The ease of producing quantities of this spirochete for *in vitro* study and the aerobic respiration of the organism make the *Leptospira* species suitable for intensive investigation of spirochetal metabolism.

In Vitro Cultivation

The nature and results of *in vitro* cultural studies have been reviewed by Soule (69), Eagle (16), Nelson (48), Stavitsky (70), Van Thiel (74), Hoffman (31), and Beerman *et al.* (2). This review will include only the relatively few studies that help to enlarge our knowledge about the *in vitro* physical and nutritional requirements for cultivation that are of value in unraveling the metabolism of pathogenic spirochetes.

Borrelia species.—The majority of attempts to cultivate the spirochetes of relapsing fever *in vitro* have made use of media containing highly complex substances, such as ascitic or hydrocele fluid, egg yolk, egg albumin, or blood (50, 35, 85, 82). No quantitative data were presented to substantiate claims for *in vitro* multiplication. These studies have done little more than confirm the fact that spirochetes of relapsing fever cannot be propagated *in vitro* by commonly-used techniques that are successful with bacteria and species of protozoa. In extensive quantitative studies in this laboratory making use of a synthetic medium simulating plasma and devised for *in vitro* cultivation of malarial parasites (1, 25), evidence for the growth of *B. novyi* was obtained, but no multiplication could be demonstrated. The growth of the spirochete by elongation together with one or two constrictions could be readily observed, but apparently those enzymes, nucleic acids, or other unknown substances necessary for division were not utilized or were not functional, if present. The extraordinary utilization of glucose by *B. novyi* cultures with the production of lactic acid sharply limits the life of cultures incubated at 37°C. Even when the diffusion technique (25) was used to maintain a pH compatible for survival, no multiplication was obtained, indicating the need for substances or conditions not provided by this method of cultivation. Since *in vitro* growth of *B. novyi* can be obtained, a plausible explanation of the failure to get *in vitro* multiplication postulates the lack in the culture mixture of nutrients, vitamins, or enzymes essential for cellular division. The unknown substances apparently exist in minute amounts in the circulating blood of the host. The substances are either oxidized or diluted out when the culture is seeded and then quickly depleted upon incubation. This theory, if substantiated by experiments with pathogenic spirochetes of the genera *Borrelia* and *Treponema*, would make these parasites dependent on the host for the supply of needed substances (enzymes, hormones, etc.) derived from active cellular metabolism in the same

way that rickettsiae are dependent on unknown substances in living or surviving cells for multiplication.

Treponema species.—To provide proper orientation in our consideration of the cultivation of *Treponema* species, the list of eight species of this genus recognized in *Bergey's Manual of Determinative Bacteriology* (3) includes both pathogenic (*T. pallidum*, *T. pertenue*, *T. carateum*, and *T. cuniculi*) and saprophytic species (*T. microdentium*, *T. mucosum*, *T. calligyrum*, and *T. genitalis*). The different habitats in this host and slight differences in morphology of these subgroups immediately indicate that they must differ also in their biochemistry and metabolism. The wide distribution of the saprophytic species, their contamination of syphilitic and yaws lesions, oral cavities, and the genitalia, and the relative ease of cultivation are the bases for the considerable confusion that exists in the literature about them. Considerable doubt has been raised about the identity of the so-called nonpathogenic species of *T. pallidum*.

Eagle & Germuth (17) made a serological study of five cultured strains of supposed *T. pallidum* (Noguchi, Kroo, Nichols, Reiter, and Kazan) and two strains of *Treponema* from the mouth in an effort to establish affinities to a strain of *T. pallidum* with demonstrable pathogenicity. The five strains of cultured nonpathogenic *T. pallidum* fell into three groups. The Noguchi and Nichols strains were serologically identical; the Reiter strain showed cross-reactions with the Kazan strain, but was not identical; and the Kroo strain was serologically distinct from the other strains. The serological identity of the Reiter strain with the two strains of saprophytic mouth species and a close relationship to the Kazan strain strongly suggested to these investigators that these organisms may have been saprophytic contaminants in the syphilitic lesion used as a source of material to inoculate cultures. A summary of this study is considered worthy of inclusion in this review not only because it helps to orient the reader, but also because it might emphasize the necessity for future investigators interested in pathogenic species of *Treponema* to use only proven strains of *T. pallidum*, *T. pertenue*, *T. carateum*, and *T. cuniculi* for their studies.

Before proceeding with the studies of Nelson (48) and Nelson & Mayer (49) and the factors affecting the survival of pathogenic *T. pallidum in vitro*, mention must be made of the findings of several groups of investigators working with the nonpathogenic Reiter strain of *T. pallidum*. These studies were carried out with the Reiter spirochete, because it can be cultured easily and it can be obtained in quantity. Little & Subbarow (38) attempted to identify the constituents of serum required by the avirulent spirochete for growth and multiplication *in vitro*. They demonstrated that serum albumin at a concentration of 0.4 to 0.5 per cent was found to be satisfactory as a substitute for serum. Other serum fractions in concentrations of 0.5 per cent were found to be inactive. The medium without the serum or albumin contained 0.8 per cent Bacto-agar, 0.5 per cent casamino acids, 0.1 per cent glucose, 0.1 per cent sodium thioglycollate, 0.01 per cent cystine, 0.025 per

cent asparagine, 0.003 per cent tryptophan, 0.5 per cent potassium phosphate, and 1 per cent of an alcohol-soluble extract of liver (50 per cent salts). The liver could be replaced in the basic medium with the addition of choline chloride, niacin, pantothenate, pyridoxine, riboflavin, and thiamine at levels of 12 mg. per cent each and ascorbic acid at 0.2 per cent.

Whiteley & Frazier (81), using the Reiter strain of *T. pallidum*, studied a variety of media for their growth-promoting properties and number of subcultures supported by each mixture. The media studied contained (*a*) an anaerobic agent and (*b*) peptone, casamino acids, casein hydrolysate or a mixture of amino acids, serum albumin, glucose, vitamins, and di-potassium phosphate. The results showed that a combination of vitamins (ascorbic acid, niacin, choline chloride, thiamine, riboflavin, pyridoxine, and calcium pantothenate) and glucose was essential for growth under anaerobic conditions in the presence of casein hydrolysate.

More extensive studies were carried out by Eagle & Steinman (19) and Steinman & Eagle (71), who showed that the nonpathogenic Reiter spirochete could be cultivated on a medium consisting of arginine, any one of a number of substances potentially capable of supplying sulfhydryl groups, crystallized serum albumin, and a small amount of Brewer's thioglycollate medium with ascorbic acid and phosphate buffer. Three components of the thioglycollate medium were considered capable of supplying nutritional factors: (*a*) trypticase and enzymatic casein digest, (*b*) yeast extract, and (*c*) glucose. It was found, however, that the enzymatic casein digest, when used along with the other substances listed above, completely replaced the thioglycollate medium and that added vitamins in glucose could then be omitted. In a series of careful cultural experiments, three of the growth factors supplied by the enzymatic casein digest were identified as pantothenic acid, glutamine, and L-phenylalanine. It was further shown that protein hydrolysate produced by acid digestion could not support growth without vitamin supplementation. The effective concentrations of the above-mentioned growth factors were 1×10^{-6} *M*, 2×10^{-3} *M*, and 1×10^{-3} *M*, respectively. As a result of these studies, the Reiter strain of spirochtees can now be grown in a chemically-defined medium, except for the necessary defined supplementation with a 0.01 per cent casein hydrolysate.

Instead of investing considerable effort in the study of avirulent *Treponema*, Nelson (48) attempted to define the factors which influence the survival of virulent *T. pallidum in vitro*. The organisms used for the study were derived from experimentally-infected rabbits. In recognition of the importance of maintaining physiological conditions throughout the course of the experiments and of using spirochetes obtained during the first appearance of clinically-recognizable syphilitic orchitis, infected testes were removed for extraction of spirochetes within 24 to 48 hr. after gross pathology could be detected. A basic medium that was chemically defined was devised to suspend the spirochetes and determine the necessity for maintaining anaerobiosis. The effects of gas phase, range of pH, temperature, and the effects

of inorganic phosphate, albumin, tissue extract, glutathione, cysteine, ascorbic acid, pyruvate, and oxaloacetate were studied. Beneficial effects were noted when 6 per cent carbon dioxide, inorganic phosphate, crystalline bovine albumin, sodium pyruvate, glutathione, cysteine, and vitamins were included in the medium. A small concentration of testicular tissue extract or serum ultrafiltrate appeared to be essential for survival of the spirochetes. No beneficial effects could be demonstrated by adding amino acids in the form of trypticase or a synthetic mixture consisting of leucine, lysine, methionine, threonine, tryptophan, valine, phenylalanine, histidine, arginine, and isoleucine. The addition of sodium acetate, *para*-aminobenzoic acid, or glucose failed to enhance survival of the spirochetes. In the most favorable mixture, motile organisms survived for six to eight days. Spirochetes showed evidence of growth, but no multiplication was obtained. The best culture medium as listed by Nelson & Mayer (49) was used to develop the immobilization test for *T. pallidum in vitro* now being elaborated upon and adapted for the practical measurement of antibody production in syphilitic infection.

From time to time, a number of investigators have tried to cultivate *T. pallidum* in tissue cultures and in developing chick embryos without any marked success. Perry (52) adapted a tissue culture method previously described for the cultivation of *Plasmodium gallinaceum* to the propagation of testicular tissue *in vitro*. Perry observed survival of *T. pallidum* for periods up to ten days, and the survival was attributed to the testicular cellular elements. No spirochetes were ever demonstrated in cultures not showing growth of testicular cells. This method deserves additional study with the use of chemically-defined media.

Leptospira species.—Species of *Leptospira*, in contrast to the genera *Borrelia* and *Treponema*, can be grown easily in liquid, semisolid, or solid media containing sodium chloride, rabbit serum, and hemoglobin. The pathogenic organisms are aerobic, growing best *in vitro* at a temperature of 25° to 27°C. Maximum growth can be obtained at a *p*H of 7.3 to 7.5, but the organisms will survive over a *p*H range of 6.4 to 8.1 according to Chang (7) and over a range of 6.0 to 8.6 according to Van Thiel (74). As stated by Stavitsky (70), these spirochetes most closely resemble bacteria in general biological properties, having a wide host range and adaptability to environmental conditions. Species of *Leptospira* are known to parasitize at least 25 species of rodents and mammals, persisting as long as 2½ years in rats (74).

With this wide range of adaptability and ease of cultivation, one would expect that a chemically-defined medium for cultivation and for study of the growth requirements and metabolism of these parasites would have been worked out. A great variety of media containing blood and tissue constituents have been successfully used but relatively few attempts have been made to devise chemically-defined media.

Rosenfeld & Greene (57) demonstrated that *L. canicola* growing *in vitro* required an unknown factor in animal (rabbit) serum and that the factor could not be replaced by nicotinic acid, thiamine hydrochloride, nicotin-

amide, riboflavin, vitamin B_6 hydrochloride, or ascorbic acid. The following substances were found to be growth-accessory factors when added to the medium containing serum: nicotinic acid, thiamine, nicotinamide, and riboflavin. Greene (29), using cultures of *L. canicola*, was unable to replace the essential serum by a series of 15 amino acids. When peptone was omitted from Schüffner's medium, partial growth was obtained with asparagine, aspartic acid, or leucine. These same substances, plus histidine and proline, gave no enhanced growth when added to the complete medium. Ten of the 15 amino acids studied showed inhibitory effects and decreasing growth when the amount of acid added was 0.025 per cent or greater. Savino & Rennella (58, 59, 60) found that a basic salt solution with the following added substances would support growth and subculture of *L. icterohaemorrhagiae:* hematin, 0.005 per cent; asparagine, 0.4 per cent; dextrin, 0.2 per cent; and a mixture of the following vitamins expressed in quantity per liter: nicotinic acid, 1 mg.; nicotinamide, 0.5 mg.; thiamine, 1 mg.; riboflavin, 0.5 mg.; pyridoxine, 0.1 mg.; pimelic acid, 50 mg.; and aspartic acid, 500 mg. Cultures without the vitamin mixture died out by the fifth subculture. Chang (8), using *L. icterohaemorrhagiae*, showed also that serum was essential for growth in culture and that the unknown substances in serum were thermolabile. Furthermore, the unknown growth factors could not be replaced by a series of vitamins, glycerol, cytochrome-*c*, and crystalline serum albumin. The enzyme poison, potassium cyanide, in an amount of 80 μg. per ml. failed to reduce growth, but iodoacetic acid in the same amount completely inhibited growth in the cultures.

Yosida (84) showed that nicotinic acid slightly stimulated the development of *L. icterohaemorrhagiae* and *L. hebdomadis*. Ward & Starbuck (77) studied the effects of nicotinic acid and cysteine hydrochloride on the growth of *L. icterohaemorrhagiae in vitro*. They showed that nicotinic acid enhanced growth in a final concentration of 0.01 per cent. Cysteine hydrochloride in the same concentration had a less marked effect, but glycine suppressed the growth of the organisms. The amino acids, valine, tyrosine, methionine, glutamine, and leucine, when added separately to cultures, failed to increase growth over that in the control cultures.

The wide experience that pathogenic species of *Leptospira* lose virulence rapidly during cultivation suggests the lack of key nutrients in commonly-used media. The studies of Chang (7) on the maintenance of virulence *in vitro* by the addition of fresh guinea pig liver to cultures bears out the above suggestion. The fact that fresh liver was necessary for the maintenance and partial survival of virulence points to the need *in vitro* for enzymes or intermediate metabolites produced by surviving cells if virulence is to be preserved.

Chemotherapy and Metabolism

The extraordinary advances in the development of successful chemotherapeutic agents in the treatment of spirochetal infections during the past

40 years have provided investigators with supplementary tools to study the metabolism of this group of parasites. The introduction of arsenical compounds for syphilotherapy beginning in 1909, of bismuth compounds by Sazarac & Levaditi in 1921 (61), which was reviewed by Goodman & Gilman (28) and Work & Work (83), and the demonstration by Mahoney *et al.* (42) that an antibiotic, penicillin, was highly effective against syphilis revolutionized the therapy of spirochetal diseases. Unfortunately, the difficulty of maintaining spirochetes *in vitro* has prevented extensive metabolic studies on the mode of action of these compounds. Consequently, ideas concerning the precise modes of action of arsenic, bismuth, mercury compounds, and antibiotics against spirochetes are little more than theories based on results obtained from studies with bacteria, protozoa, and tissue cells. The experiments of Voegtlin, Dyer, & Leonard (76) suggesting that arsenical drugs combine with sulfhydryl groups in the cell and inhibit essential metabolic processes verified a similar theory advanced by Ehrlich & Hata in 1909 (20). Hawking (30) studied the chemotherapeutic activity of a series of organic trivalent arsenic compounds and of diaminomethyl acridine upon relapsing fever spirochetes *in vitro* and upon trypanosomes (*T. rhodesiense*). The experiments were carried out over a period of 24 hr., the effects of the drugs being judged by the motility of surviving organisms. The surprising observation that some of the spirochetes resisted a concentration of drug eight times as great as that which kills the majority is in keeping with the apparent adaptability of a small percentage of spirochetes for tissue invasion, survival, and reinvasion of the blood stream to produce relapse. The approximate parallelism of the drugs tested upon spirochetes and upon trypanosomes was shown by the spirochetes requiring approximately 20 times the concentration of drug as that required to produce death of trypanosomes. No attempts were made by this investigator to study the biochemical effects of the drugs tested on the spirochetes.

Eagle (12) studied the *in vitro* action of bismuth compounds on the pathogenic Nichols strain and three newly-isolated strains of *T. pallidum*. Criteria for *in vitro* activity were immobilization and viability of the organisms as determined by the development of infection in rabbits inoculated with the test suspensions. These experiments showed that the bismuth compounds tested killed *T. pallidum in vitro*, but that the compounds were approximatley 1/4 to 1/28 as active as "arsenoxide" when compared on a molar basis. Tissue extracts inhibited the spirocheticidal action of bismuth instead of enhancing the action. The conclusion was reached from these experiments that

> bismuth compounds act neither as catalysts, inhibitors, nor precursor substances, but that the therapeutic action of bismuth in syphilis depends in fact, and perhaps primarily, on its direct spirocheticidal action on *Sp. pallida*.

Subsequently, Eagle (13, 14, 15) showed that molecular oxygen had no *in vitro* effect on the lethal action of "arsenoxide" and bismuth compounds on

pathogenic strains of *T. pallidum*. Methionine and the sulfhydryl compounds (cysteine, glutathione, and thioglycollic acid) when added in sufficient amounts to mixtures containing arsphenamines ("arsenoxide") and bismuth or mercury compounds almost completely inhibited this lethal action *in vitro*. These studies are consistent then with the theory that the heavy metals act on pathogenic spirochetes as a result of their affinity for sulfhydryl-containing compounds. *In vitro* biochemical experiments with spirochetes are needed, however, to determine the mechanism of these lethal effects.

Experimental studies with antibiotics have been done *in vivo* with relapsing fever, syphilis and yaws in rabbits, and Weil's disease, and *in vitro* on surviving *T. pallidum*, on cultures of the Reiter strain of *Treponema*, and on cultures of *L. icterohaemorrhagiae*. The *in vivo* studies were carried out to determine the therapeutic properties of the antibiotics—penicillin, chloramphenicol, aureomycin, terramycin, bacitracin, and subtilin. Relatively few *in vitro* studies have been done with spirochetes, and the results of these experiments have been interpreted primarily by effects on viability and morphology, rather than on the biochemistry or metabolism of the organisms. Since more information is available about the mode of action of penicillin on infectious agents and disease, the following discussion will deal mainly with this antibiotic.

The rapid superficial disappearance of spirochetes from lesions of syphilis, yaws, and pinta within 24 hr. after a therapeutic dose of penicillin and the spirocheticidal action of this antibiotic on organisms *in vitro* is evidence of positive, though partially delayed, action. The slow rate of multiplication, generation times of approximately 30 hr. for *T. pallidum* in the host (41) and 8.8 hr. for the Reiter spirochete *in vitro* (26), makes these organisms extremely vulnerable to drug action during their long growth phase. Eagle & Musselman (18) demonstrated that penicillin exerted spirocheticidal effects *in vitro* against four strains of nonpathogenic *T. pallidum* and a strain of mouth spirochetes. The effective threshold concentration was 0.01 unit per ml. with activity increasing up to 0.1 to 0.25 unit per ml., but higher concentrations failed to accelerate effects. Motility of the organisms continued for 8 to 24 hr. after exposure to penicillin. The rate at which the organisms were killed increased with temperature within the range of 8° to 40°C. Tung & Frazier (72) observed that spirochetes of the Reiter strain when exposed in culture to penicillin developed into giant elongated organisms two to eight times their normal length (20μ) and without their terminal flagellae as determined with the aid of an electron microscope. These long forms appeared after 24 hr. of incubation and disappeared gradually after 72 hr. Frazier & Frieden (23) observed similar morphological changes of *T. pallidum* in man after treatment with penicillin was initiated. Nelson (48), using the basal medium referred to above, tested the effects of crystalline sodium penicillin G on *T. pallidum* from experimental rabbit syphilis. Immobilization reached a 50 per cent end point at 12 hr. and a 90 per cent end point at

24 hr. No morphological changes were noted. Graded effects occurred with .001 to 0.1 μg. per ml., but higher concentrations did not increase the rate of immobilization.

Chang (6) studied the effect of penicillin on *L. icterohaemorrhagiae* in culture and in guinea pigs and demonstrated that 0.4 Oxford unit per ml. of medium or 0.2 Oxford unit per ml. of serum were necessary to obtain stasis of growth and that there was no advantage in increasing the amount of the antibiotic. Multiplication was prevented in the presence of penicillin, and the spirochetes survived only a limited number of days.

By analogy, the morphological effects of penicillin on susceptible spirochetes agree with similar effects observed with bacteria (55). No biochemical effects of penicillin on spirochetes of the type shown for gram positive bacteria by Gale and his collaborators (24) have been reported. These workers suggest that penicillin prevents the assimilation of amino acids, in particular glutamic acid, by gram positive organisms. Hotchkiss (32, 33), using different experimental procedures, obtained results that suggested penicillin interference with or blockage of protein synthesis from amino acids by bacteria and accumulation of peptide intermediates instead. Only *in vitro* experiments will determine the biochemical mechanism by which penicillin exerts its effects on susceptible spirochetes. The spirochetes are, however, gram-negative and Gale (24) points out that gram negative organisms do not possess the ability to concentrate amino acids in the internal environment. Action by penicillin on gram negative spirochetes might differ, but the studies reviewed above are in agreement at least with current theories (24, 5) that penicillin exerts its action on growing rather than on resting organisms and that cell division and possibly nucleic acid metabolism might be affected.

Of considerable interest are the cytochemical studies of Pratt & Dufrenoy (55), indicating that penicillin exerts its effects by dehydrogenation of —SH groups to S—S more rapidly than the active —SH groups are restored. This attack on the vulnerable —SH groups by two chemically different groups of compounds, the heavy metals and antibiotics, deserves intensive comparative studies on susceptible spirochetes, on spirochetes that react differently to arsenicals and antibiotics, and finally on organisms such as pathogenic trypanosomes that are affected by arsenicals and not by penicillin. Reports that terramycin exceeds the activity of other antibiotics against syphilis and yaws should stimulate further investigation into comparative modes of action.

The variable results obtained with penicillin in curing relapsing fever and long-standing syphilitic infections might be explained by the failure of the antibiotic to penetrate the central nervous system, by the existence of permanent tissue damage or low vascular supply in tissues caused by the infection, or by the parasite's lack of active growth because of host-immune responses. No evidence of the development of strains of spirochetes resistant to the action of antibiotics has been presented.

Discussion and Conclusion

The inability to grow disease-producing *Borrelia* and *Treponema in vitro* might easily be the result of failure to provide an environment and substrates essential for multiplication and required for the elaboration of those enzymes that play a part in their pathogenic mode of life. As Dubos (11) points out, many metabolic phenomena unrelated to host-immune reactions play a part in pathogenicity of bacteria, and this statement is equally applicable to our interpretation of spirochetal behavior in man, in experimental animals, in reservoir hosts, or in the transmitting vectors. The rapid loss of pathogenicity by cultured strains of *Leptospira;* the failure up to this time to get multiplication of the spirochetes of relapsing fever, syphilis, and yaws *in vitro;* the different survival properties of *T. pallidum* when taken from lesions of experimental syphilis in rabbits during the edematous and inflammatory stage of the infection, versus the late syphilomatous stages; and the differential *in vitro* metabolic behavior of *Borrelia novyi* taken from the blood of rats near the crisis of the primary infection, versus those taken earlier in the infection, all indicate the operation of subtle metabolic phenomena that will require exacting study of these parasites before adequate explanations of their metabolic activities can be elicited. The effects of slight and often minute changes in the composition of a medium on toxin production by bacteria (diphtheria bacilli) and on virulence (culture of capsulated pneumococci) pose difficult but not insurmountable problems for the investigator of spirochetal metabolism.

LITERATURE CITED

1. Anfinsen, C. B., Geiman, Q. M., McKee, R. W., Ormsbee, R. A., and Ball, E. G., *J. Exptl. Med.*, **84**, 607–21 (1946)
2. Beerman, H., Nicholas, L., Buerk, M. S., and Ford, W. R., *Arch. Internal Med.*, **85**, 305–58, 496–541, 699–721, 819–86 (1950)
3. Bergey, D. H., *Bergey's Manual of Determinative Bacteriology* (Williams & Wilkins Co., Baltimore, Md., 1529 pp., 1948)
4. Bertucci, E. A., Jr., *Am. J. Med. Sci.*, **209**, 86–111 (1945)
5. Brownlee, G., *Ann. Rev. Microbiol.*, **5**, 197–208 (1951)
6. Chang, S. L., *J. Clin. Invest.*, **25**, 752–60 (1946)
7. Chang, S. L., *J. Infectious Diseases*, **81**, 28–34 (1947)
8. Chang, S. L., *J. Infectious Diseases*, **81**, 35–47 (1947)
9. Davis, G. E., *Ann. Rev. Microbiol.*, **2**, 305–34 (1948)
10. Dempsey, E. W., and Wislocki, G. B., *Physiol. Revs.*, **26**, 1–27 (1946)
11. Dubos, R. J., *Bact. Revs.*, **12**, 173–94 (1948)
12. Eagle, H., *Bull. Johns Hopkins Hosp.*, **63**, 305–17 (1938)
13. Eagle, H., *J. Pharmacol. Exptl. Therap.*, **64**, 164–89 (1938)
14. Eagle, H., *J. Pharmacol. Exptl. Therap.*, **66**, 423–35 (1939)
15. Eagle, H., *J. Pharmacol. Exptl. Therap.*, **66**, 436–48 (1939)
16. Eagle, H., in *Bacterial and Mycotic Infections of Man*, 527–55 (Dubos, R. J., Ed., J. B. Lippincott Co., Philadelphia, Pa., 785 pp., 1948)
17. Eagle, H., and Germuth, F. G., Jr., *J. Immunol.*, **60**, 223–39 (1948)
18. Eagle, H., and Musselman, A. D., *J. Exptl. Med.*, **80**, 493–505 (1944)

19. Eagle, H., and Steinman, H. G., *J. Bact.*, **56**, 163–76 (1948)
20. Ehrlich, P., and Hata, S., *The Experimental Chemotherapy of Spirilloses* (Rebman Co., New York, N. Y., 181 pp. (1911))
21. Fenyvessy, B. v., and Scheff, G., *Biochem. Z.*, **221**, 206–16 (1930)
22. Fischer, A., in *Experimental Cell Research*, 430–36 (Proc. 6th Intern. Congr. Exptl. Cytology, Albert Bonnier, Stockholm, Sweden, 603 pp., 1949)
23. Frazier, C. N., and Frieden, E. H., *J. Am. Med. Assoc.*, **130**, 677–83 (1946)
24. Gale, E. F., *Symposia Soc. Exptl. Biol.*, **3**, 233–42 (1949)
25. Geiman, Q. M., Anfinsen, C. B., McKee, R. W., Ormsbee, R. A., and Ball, E. G., *J. Exptl. Med.*, **84**, 583–606 (1946)
26. Gelperin, A., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **33**, 101–13 (1949)
27. Glick, D., *Techniques of Histo- and Cytochemistry* (Interscience Publishers, Inc., New York, N. Y., 531 pp., 1949)
28. Goodman, L., and Gilman, A., *The Pharmacological Basis of Therapeutics* (The Macmillan Co., New York, N. Y., 1387 pp., 1941)
29. Greene, M. R., *J. Bact.*, **50**, 39–45 (1945)
30. Hawking, F., *Ann. Trop. Med. Parasitol.*, **33**, 1–11 (1939)
31. Hoffman, H., *Oral Surg.*, **2**, 925–38 (1949)
32. Hotchkiss, R. D., *Ann. N. Y. Acad. Sci.*, **53**, 13–17 (1950)
33. Hotchkiss, R. D., *Federation Proc.*, **6**, 263 (1947)
34. Kinsey, V. E., *J. Gen. Physiol.*, **34**, 389–402 (1951)
35. Kirk, R., *Ann. Trop. Med. Parasitol.*, **32**, 342–43 (1932)
36. Krebs, H. A., *Ann. Rev. Biochem.*, **19**, 409–30 (1950)
37. Krebs, H. A., *Biochim. et Biophys. Acta*, **4**, 249–69 (1950)
38. Little, P. A., and Subbarow, Y., *J. Immunol.*, **50**, 213–19 (1945)
39. Lofgren, R., and Soule, M. H., *J. Bact.*, **50**, 679–90 (1945)
40. Lwoff, A., in *Biochemistry and Physiology of Protozoa*, 1–26 (Andre Lwoff, Ed., Academic Press, Inc., New York, N. Y., 434 pp., 1951)
41. Magnuson, H. J., Eagle, H., and Fleishman, R., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **32**, 1–18 (1948)
42. Mahoney, J. F., Arnold, R. C., and Harris, A., *Venereal Disease Inform.*, **24**, 355–57 (1943)
43. Marshall, P. B., *J. Infectious Diseases*, **84**, 150–52 (1949)
44. McKee, R. W., and Geiman, Q. M., *Federation Proc.*, **9**, 201–2 (1950)
45. McKee, R. W., and Geiman, Q. M. (Unpublished data)
46. Most, H., Ed., *Parasitic Infections in Man* (Columbia Univ. Press, New York, N. Y., 229 pp., 1951)
47. Mudd, S., Polevitzky, K., and Anderson, T. F., *J. Bact.*, **46**, 15–24 (1943)
48. Nelson, R. A., Jr., *Am. J. Hyg.*, **48**, 120–32 (1948)
49. Nelson, R. A., Jr., and Mayer, M. M., *J. Exptl. Med.*, **89**, 369–93 (1949)
50. Noguchi, H., *J. Exptl. Med.*, **16**, 199–210 (1912)
51. Noguchi, H., in *The Newer Knowledge of Bacteriology and Immunology*, 452–97 (Jordan, E. O., and Falk, I. S., Ed., Univ. of Chicago Press, Chicago, Ill., 1196 pp., 1928)
52. Perry, W. L. M., *J. Path. Bact.*, **60**, 339–42 (1948)
53. Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, **1**, *Interpretations* (Williams & Wilkins Co., Baltimore, Md., 1041 pp., 1946)
54. Porter, J. R., *Bacterial Chemistry and Physiology* (John Wiley & Sons, Inc., New York, N. Y., 1073 pp., 1946)
55. Pratt, R., and Dufrenoy, J., *Bact. Revs.*, **12**, 79–103 (1948)

56. Robinow, C. F., Addendum to *The Bacterial Cell*, 353–77 (R. Dubos, Harvard Univ. Press, Cambridge, Mass., 460 pp., 1946)
57. Rosenfeld, W. D., and Greene, M. R., *J. Bact.*, **42**, 165–72 (1941)
58. Savino, E., and Rennella, E., *Rev. soc. argentina biol.*, **18**, 176–89 (1942)
59. Savino, E., and Rennella, E., *Rev. soc. argentina biol.*, **18**, 566–78 (1942)
60. Savino, E., and Rennella, E., *Rev. soc. argentina biol.*, **18**, 579–83 (1942)
61. Sazerac, R., and Levaditi, C., *Compt. rend.*, **173**, 1201–4 (1921)
62. Scheff, G., *Zentr. Bakt. Parasitenk.*, **134**, 35–42 (1935)
63. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83–89 (1945)
64. Schuhardt, V. T., *Pub. Am. Assoc. Advance Sci.*, No. 18, 58–66 (1942)
65. Scott, V., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **33**, 424–28 (1949)
66. Scott, V., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **34**, 12–17 (1950)
67. Scott, V., Dammin, G., and Droegemueller, C., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **34**, 501–14 (1950)
68. Scott, V., and Droegemueller, C., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **34**, 1–11 (1950)
69. Soule, M. H., *Pub. Am. Assoc. Advance. Sci.*, No. 18, 53–57 (1942)
70. Stavitsky, A. B., *Bact. Revs.*, **12**, 203–55 (1948)
71. Steinman, H. G., and Eagle, H., *J. Bact.*, **60**, 57–68 (1950)
72. Tung, T., and Frazier, C. N., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **30**, 205–10 (1946)
73. Turner, T. B., and Hollander, D. H., *Bull. Johns Hopkins Hosp.*, **87**, 505–9 (1950)
74. Van Thiel, P. H., *The Leptospiroses* (Universitaire pers Leiden, Leiden, The Netherlands, 231 pp., 1948)
75. Van Thiel, P. H., and Van Iterson, W., *Proc. Koninkl. Nederland. Akad. Wetenschap.*, **50**, 976–79 (1947)
76. Voegtlin, C., Dyer, H. A., and Leonard, C. S., *U. S. Pub. Health Repts.*, **38**, 1882–1912 (1923)
77. Ward, T. G., and Starbuck, E. B., *Proc. Soc. Exptl. Biol. Med.*, **48**, 19–21 (1941)
78. Wenyon, C. M., *Protozoology* (William Wood & Co., New York, N. Y., 2 vols., 1563 pp. 1926)
79. Werkman, C. H., and Wilson, P. W., Eds., *Bacterial Physiology* (Academic Press, Inc., New York, N. Y., 707 pp., 1951)
80. White, P. R., *Growth*, **10**, 231–89 (1946)
81. Whiteley, H. R., and Frazier, C. N., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **32**, 43–52 (1948)
82. Wolman, B., and Wolman, M., *Ann. Trop. Med. Parasitol.*, **39**, 82–93 (1945)
83. Work, T. S., and Work, E., *The Basis of Chemotherapy* (Interscience Publishers, Inc., New York, N. Y., 435 pp., 1948)
84. Yosida, N., *Fukuoka Acta Med.*, **32**, 88–89 (1939)
85. Yuan-Po, L., *Kitasato Arch. Exptl. Med.*, **10**, 78–86 (1933)

RECENT ADVANCES IN THE TAXONOMY OF YEASTS[1]

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This review will deal exclusively with the limited field stated in the title. Related areas of classification procedures, including cytology, genetics, ecology, and the relationships to other fungi will be mentioned only incidentally. In no wise will it be a compilation of papers describing miscellaneous new species or treating minor phases of classification. Rather, it will be held to a discussion of those contributions which in the judgement of the reveiwer may have some continuing influence on concepts of yeast taxonomy.

The treatment given to contributions will be proportional to their anticipated impact on yeast classification, which means that in essence this review will be concerned with the current book by Lodder & Kreger-van Rij (17) respectively of the Gist- en Spiritusfabriek N. V., and the Yeast Division, Centraalbureau voor Schimmelcultures, Delft, Holland. The works of other contributors will largely be fitted into the text at proper places. Thus, Etchells & Bell's work (9) on *Brettanomyces* Kufferath et Van Laer will be discussed together with the Dutch workers' version of that genus and Wickerham's (31) work on *Hansenula* H. et P. Sydow will be discussed with the Dutch authors' version of *Hansenula*. Such organization seems simple and proper, for the Dutch views will constitute standards with which, or away from which, future contributions will coincide or deviate. The role of the reviewer will be to discuss the value of the contributions as he sees them, and to make suggestions, being cognizant that some of them eventually may be proven ill-advised.

Dr. Lodder and Mrs. Kreger-van Rij have very kindly made available a typewritten copy of their book, which is expected to be in print by May, 1952, and should therefore be available when this review is off the presses. Their kindness is greatly appreciated.

The techniques used by the Dutch investigators are essentially the same as those used by Diddens & Lodder (7). Because many readers may not be acquainted with the changes made in this new book, the older and more common species epithets will be used in this review except where it is obvious from the text that new names are being used.

The Dutch workers divide the yeasts into three groups, namely the families Endomycetaceae, Sporobolomycetaceae, and Cryptococcaceae.

The Endomycetaceae includes the yeasts which form ascospores. It also includes two genera, *Eremascus* Eidam and *Endomyces* Reess, which are not considered by Lodder & Kreger-van Rij to be yeasts, because *Eremascus*

[1] This report covers the period 1948 to 1952.

forms only true mycelium and no yeast cells, and *Endomyces* is characterized by a true mycelium which breaks up into arthrospores, but particularly because in both genera ascospores are produced in specialized cells referred to as gametangia. *Eremascus* is usually considered to be the progenitor of most of the ascosporogenous yeasts.

Schizosaccharomyces Lindner is a small genus in which asexual reproduction is exclusively by fission, and asci are formed by the fusion of ordinary arthrospores. The genus has been broadened in concept to include species producing hyphae, this change being required to permit inclusion of *S. versatilis* Wickerham et Duprat (34). *Schizosaccharomyces japonicus* Yukawa et Maki (38), which was discovered earlier, is given as a questionable synonym. It is the reviewer's opinion that although the two yeasts are very similar, species rank is justified for each, because *S. versatilis* produces reniform spores and *S. japonicus* produces round or oval spores.

However, should other taxonomists believe that the slight difference in spore shape is insufficient grounds for separation, the writer believes the correct name, on the basis of priority, should be *S. japonicus*, even though a more accurate description of the species was given in the original description of *S. versatilis*. For persons who may wish to study sporulation of these species, we have found useful Dr. Francis M. Clark's suggestion (personal communication) that 5 per cent glucose be used in the medium, and incubation may well be at 28°C. The conditions are given here because they are somewhat unusual for yeasts of other genera. Stains may be better for determining shape of spores in these species, since reniform spores when dried in films tend to lie on their side, whereas cells in wet mounts may float bottoms up and show an ellipsoidal shape.

The distinction between the genera *Endomyces*, which reproduces asexually only by arthrospores, and *Endomycopsis* Dekker, which reproduces asexually only by blastospores, has been made clearer by removal of *Endomycopsis vernalis* (Ludwig) Stelling-Dekker to *Trichosporon* Behrend, where it appears as a synonym for *T. pullulans* (Lindner) Diddens et Lodder. This species produces both blastospores and arthrospores. Lodder & Kreger-van Rij (17) believe that it probably did not produce ascospores even when first isolated. The definition of the genus *Endomycopsis* has been expanded to include species which assimilate nitrate. *Endomycopsis albicans* (Vuill.) Dekker has been weeded out and now appears as a synonym for *Candida albicans* (Robin) Berkhout. Thus the Dutch workers have continued the taxonomic clarification started by Stelling-Dekker's cleavage of the genus *Endomyces* Reess into *Endomyces* and *Endomycopsis* (29).

The subgenera *Zygosaccharomyces* Barker, *Zygopichia* Klöcker, and *Zygohansenula* Lodder are no longer recognized; the species formerly included now are placed respectively in *Saccharomyces* (Meyen) Reess, *Pichia* Hansen, and *Hansenula* H. et P. Sydow. Various investigators, including Winge & Laustsen (37) and Lindegren & Lindegren (14), have demonstrated that no fundamental difference exists between the type of sexual reproduction shown by species of *Saccharomyces* and *Zygosaccharomyces*. Lodder (16)

suggested merging these subgenera as a result of clear and convincing evidence. She found that the species *Saccharomyces marxianus* Hansen exists in some strains exclusively as diploid vegetative cells. When these sporulate the ascus is, of course, a single cell. Other strains exist partially or completely as haploid cells, and therefore some or all of the asci are conjugated cells. Formerly, strains which produced asci that consisted of a single cell were placed in *Saccharomyces*, and those which produced asci that consisted of two fused cells were placed in *Zygosaccharomyces*. In *S. marxianus*, the tendency to exist in either the haploid or the diploid phase is so delicately balanced that by selecting the right medium either the diplophase (*Saccharomyces*) or the haplophase (*Zygosaccharomyces*) may be maintained, and either phase can, by appropriate procedure, be converted to the other.

Despite the merger of *Zygosaccharomyces* and *Saccharomyces* by Lodder & Kreger-van Rij (17), the enlargement of the genus has not been excessive, for the number of species of osmophilic *Zygosaccharomyces* previously recognized has been greatly reduced. A reduction in this area has been desired by some zymologists for many years. The fermentative species of *Debaryomyces* Klöcker have been transferred to *Saccharomyces* and the value of apparently rough walls as a generic characteristic has been de-emphasized. However, *Debaryomyces* is maintained for the nonfermentative yeasts customarily placed in this genus. *Torulaspora* Lindner has been put *in toto* into *Saccharomyces*. This reviewer believes the Dutch action denoting a close relationship between *Debaryomyces* and *Torulaspora* is a forward step on the long, rough road that eventually will lead to a satisfactory classification of the yeasts. It is also a pleasure to note that the number of varieties previously appended to various species has been markedly reduced.

Closer inspection of synonymy of some species raises questions. The manuscript of Lodder & Kreger-van Rij lists *Debaryomyces globosus* Klöcker as a possible synonym of *Saccharomyces rosei* (formerly *Torulaspora rosei* Guilliermond). They state that the two strains of *D. globosus* available to them did not sporulate; but if a study of other cultures should show the ascospores to be rough, *D. globosus* should be considered as a separate species. This reviewer has noted the marked roughness of the ascospore walls in several strains of *D. globosus* isolated from soils, and he has sent two of these to the Centraalbureau in the rather faint hope that the yeasts would arrive in time to permit a change in their book, should the authors find it desirable to do so.

Holst's *Zygosaccharomyces pini* (11) has been placed in synonomy with *Zygosaccharomyces pastori* Guilliermond. It is not surprising that the Dutch workers would so regard Holst's species, because of the limited number of carbon compounds which they use in taxonomic studies, and because glucose is the only sugar fermented by this yeast. However, in consideration of seven carbon compounds which *Z. pini* can assimilate, but which *Z. pastori* cannot, the reviewer definitely considers them to be separate species. To place differently named strains of a single species in synonomy, after study by adequate techniques, is of course commendable, and in this regard three

generations of zymologists at the Centraalbureau have excelled; but the placement of different species in synonomy because adequate techniques were not used, is unfortunate. Similar cases are not uncommon for other species that ferment few or no sugars.

Etchells & Bell (8) have described an interesting species which they named *Zygosaccharomyces halomembranis* because it rapidly produces luxuriant films on media containing 5 to 20 per cent salt, though film formation is negligible to absent in liquid media without salt. Film formation does not occur with ethyl alcohol as a source of carbon, although growth is good. This yeast is interesting because species of *Zygosaccharomyces* are generally considered incapable of forming prominent pellicles.

Wiles (35) found that most of the yeasts causing spoilage of English draft beers were species of *Saccharomyces*. These, by comparison with descriptions contained in Stelling-Dekker's monograph (29) and with representative strains, were identified as *S. cerevisiae* var. *turbidans* (Hansen) Dekker and *S. carlsbergensis* Hansen. The strains placed in the latter species showed a gradation of slight morphological differences, some being typical of *S. carlsbergensis*, while others were more closely related to *S. validus* Hansen and *S. pastorianus* Hansen. The cultures most closely resembling *S. pastorianus* were obtained from old slants and colony sectors of a particular strain of *S. carlsbergensis*. Wiles believes he has a good case for recommending the inclusion of *S. carlsbergensis*, *S. validus*, and *S. pastorianus* as varieties within a single species. It is of interest to note that Lodder & Kreger-van Rij have maintained *S. carlsbergensis* and *S. pastorianus* as separate species, but *S. validus* has been placed partly in *S. willianus* Saccardo and partly in *S. uvarum* Beijerinck, depending upon the ability of individual strains to ferment melibiose.

A perusal of *Pichia* reveals again the fact that Lodder & Kreger-van Rij (17) have lumped much more than they have split. *Pichia mandshurica* Saito, *P. alcoholophila* Klöcker, *P. belgica* (Lindner) Dekker, and *P. chodati* (Zender) Dekker, which were recognized by Stelling-Dekker (29) as valid species among the six species which she included in the genus, are not recognized by Lodder & Kreger-van Rij. The four species recognized by the latter authors are *P. membranaefaciens* Hansen, the type species; *P. farinosa* (Lindner) Hansen, which was taken from *Zygopichia; P. fermentans* Lodder, a relatively new species; and *P. polymorpha* Klöcker.

Of the five species of *Hansenula* recognized by Stelling-Dekker in this nitrate-assimilating genus of budding yeasts, four were maintained, and the fifth, *H. javanica* (Groenewege) Dekker, was placed in synonomy with *H. anomala* (Hansen) Sydow. All varieties of *H. anomala* previously recognized by Stelling-Dekker and by Bedford (2) were eliminated because the morphological differences formerly separating the varieties were very gradual and, moreover, variable. *Hansenula ciferri* Lodder was lowered in rank to a variety of *H. anomala*. The single species in *Zygohansenula* Lodder, *Z. californica* Lodder, was transferred to *Hansenula*.

Three new species described by Wickerham (31) were accepted; namely,

H. minuta, *H. mrakii*, and *H. silvicola*. Unfortunately, *H. capsulata*, *H. canadensis*, and *H. angusta*, three additional new species described by the writer, were sent to Lodder and Kreger-van Rij too late to be considered in their book. Discovery by Wickerham of species of heterothallic *Hansenula* which exist exclusively as haploid yeasts in their natural vegetative stage was made too late to be included in his own publication (31). One of those species will be discussed later in this review.

Wickerham (31) recognized *H. ciferri* and *H. jadinii* (S., S., W., et M.) Wickerham as distinct species. *Hansenula ciferri* is not regarded as a variety of *H. anomala* because it produces variants that form abundant true hyphae with clearly apparent septa, and it assimilates rhamnose, two abilities not found in any of the more than 70 strains of *H. anomala* he studied.

Hansenula jadinii is placed by the Dutch workers in *H. subpelliculosa* Bedford, despite the following characteristics, all of which are routinely studied by the reviewer but not by the Dutch workers: Assimilation of erythritol, a very important carbon source for the separation of species in this genus, differs for the two species; all strains of *H. subpellicolosa* are osmophilic, *H. jadinii* is not; and *H. subpelliculosa* does not grow in a chemically-defined medium devoid of vitamins, although *H. jadinii* does.

A further divergence of opinions centers about *H. beckii* (Beck) Wickerham. The Dutch workers want it maintained as *Endomycopsis bispora* (Beck) Dekker because it produces an abundance of true hyphae; the writer placed it in *Hansenula* because he believes that the separation of genera on the basis of the ability to produce true hyphae should be avoided where its use would separate species that are obviously closely related. He believes that in *Hansenula*, and probably in other genera of yeasts, phylogenetic lines can be made rather clearly discernible; and that each such line should be defined as a genus or group of genera, without regard to the now generally accepted generic characteristics such as hyphal formation, the ability to produce a gaseous fermentation, or to produce pellicles. Evolutionary development in *Hansenula* may be shown by a gradual transition from species to species or among groups of species by such characteristics as the ratio of cells with haploid to diploid nuclei in vegetative cultures, the size of the cells, the ability to produce pellicles, the formation of esters, the rate of fermentation, the number of sugars fermented, and the dependence upon an external source of vitamins for growth. A simple visual means of arranging the species according to phylogenetic development in *Hansenula* is to place streak plates of all the species on a table. The transition from completely haploid species to completely diploid species is shown by a gradual change from mucoid, to submucoid, then to glistening and butyrous, and finally to mat colonies. The generic characterization of *Hansenula* is simple: reproduction asexually by budding, formation of one to four ascospores per ascus, and assimilation of nitrate. It remains, of course, to be seen how well the writer can work out what he considers to be phylogenetic lines for the remaining genera of yeasts and how well he can define those genera.

Very little change has been made by Lodder & Kreger-van Rij (17) in

the small genus *Saccharomycodes* Hansen which reproduces asexually by a process intermediate between fission and budding. The number of species in *Hanseniaspora* Zikes, in which budding is on a broad base, has been reduced to one, *H. valbyensis* Klöcker. The apiculate yeasts which produce a single round ascospore are not recognized because the Dutch authors do not believe the ascospore-producing ability has been definitely established. In the genus *Nadsonia* Sydow, *N. richteri* Kostka has been reduced to synonomy with *N. elongata* Konokotine.

Little change has been made in the subfamily Nematosporoideae, characterized by needle-shaped ascospores, except for the genus *Nematospora* Peglion which the Dutch now regard as containing only a single species, *N. coryli* Peglion. The genus *Ashbya*, tentatively included by Stelling-Dekker, is excluded from the ascosporogenous yeasts because its spore sac is believed not to be an ascus, and because it forms budding cells rarely.

This reviewer is also of the opinion that *Ashbya* is just outside the boundary limits of the yeasts, but is of prime interest as a near relative of the genus *Nematospora*. There probably are too many genera in the region of *Ashbya*, *Eremothecium*, and *Spermophthora*. Possibly these three genera should be reduced to the one having the earliest valid name. The species *E. ashbyii* Guilliermond and *A. gossypii* (Ashby et Nowell) Guill. are closely related indeed, the only difference of sufficient taxonomic value to separate them into species being the shape of the ascospores. It is likely that *Spermophthora gossypii* is also very similar, the main difference being, as in the subgenera *Zygosaccharomyces* and *Saccharomyces*, the time at which diploidization takes place. It is now common knowledge that diploidization in different strains of the same species may take place when the ascospores germinate, or during the period of vegetative growth, or at the end of the vegetative phase. It is possible that a similar situation holds for the cotton pathogens, *Ashbya*, *Eremothecium*, and *Spermophthora*. Recently Routien (22) has submitted another name, *Crebrothecium*, to replace *Eremothecium*. What this field needs is fewer names and more intensive study of the sexuality of many strains, just after they have been freshly isolated from nature as well as at a later date, to learn what modifications may occur in the nuclear cycle during long maintenance on laboratory media. The results would be of value, the writer believes, in showing the diversity of nuclear procedures possessed by a very few species in a single genus, rather than a diversity of species in three genera.

Lodder & Kreger-van Rij (17) have created a new genus of ascosporogenous yeasts, *Lipomyces*, and a new subfamily for it. In it are placed the former *Torulopsis lipofera* (den Dooren de Jong) Lodder, and Starkey's (28) fat yeast. The main characteristic of the new genus is the way the asci are formed. A vegetative cell, which contains a large fat globule, produces a sac-like protuberance which becomes the ascus. Granular material forms in the ascus and then becomes transformed into ascospores. The ascus usually remains attached to the mother cell, which may bear additional asci or buds. The number of ascospores is variable, ranging from 4 to 16 or more. The smooth, oval spores have a light amber color. Sugars are not fermented.

The two species, *L. starkeyi* Lodder et Kreger-van Rij and *L. lipoferus*, are separated on the tenuous grounds of slight differences in the shape of vegetative cells and the assimilation of lactose.

The genus *Saccharomycopsis* Schiönning, with the single species *S. guttulatus* (Robin) Schiönning, is tentatively accepted by Lodder and Kreger-van Rij. Its natural habitat is the stomach and intestines of rabbits, and it has been found in some other animals. The fact that it cannot be maintained on solid media should be a challenge to physiologically-minded zymologists.

The family Sporobolomycetaceae is characterized by the production of ballistospores. The Dutch taxonomists are of the opinion that too little information is yet at hand to permit the family to be correctly placed either among the Basidiomycetes or the Fungi Imperfecti, since they contend no sexual process has been demonstrated in the formation of the ballistospores. Of the four genera placed in the family by Derx (6), *Sporobolomyces* Kluyver et van Niel containing seven species and *Bullera* Derx containing two species are considered as yeasts because vegetative reproduction is mainly by budding. *Tilletiopsis* Derx and *Itersonilia* Derx do not produce yeast cells; they form true mycelium exclusively and are, therefore, excluded. Lodder & Kreger-van Rij doubt whether they should be placed in the Sporobolomycetaceae.

The spores of *Sporobolomyces* are asymmetrical, kidney-shaped or sickle-shaped, and the color of the cultures is red to salmon-pink. *Bullera* has symmetrical, round to oval spores, and the color of the cultures ranges from pallid to yellowish. The inclusion of these genera may not be hailed with joy by all zymologists. Some may consider the relationships of the groups within the ever-broadening boundaries of the yeasts as too remote to consider them as a single area of fungi. On the other hand, from a purely practical view, their placement with the yeasts will submit them to study by zymologists who otherwise might not study them. This is good, for we shall never be certain that the yeasts are well classified until all the related or seemingly related genera likewise have been thoroughly studied, preferably by persons who know yeasts as well as such other forms as *Tilletiopsis*, *Itersonilia*, *Taphrina*, *Dipodascus*, *Ashbya*, the smuts, the dematiaceous yeast-like forms, and all the other groups that may or may not contain truly related forms.

Lodder & Kreger-van Rij (17) have placed all of the yeasts which form neither ascospores nor ballistospores in the family Cryptococcaceae. The reviewer will discuss more or less together the genera *Candida* Berkhout, *Cryptococcus* Kützing emend. Vuillemin, *Rhodotorula* Harrison, *Torulopsis* Berlese, and *Trichosporon*.

Cryptococcus contains a rather small number of nonfermentative budding yeasts which synthesize starch but do not produce hyphae. They assimilate nearly all of the carbon compounds ordinarily used in taxonomic studies, and variable reactions are rather common. A very wide range of assimilation reactions is also found for the starch-producing species of *Trichosporon* and *Candida* and for most of the species of *Rhodotorula*. The use of a limited number of carbon sources by the Dutch workers leaves little basis upon which to

differentiate the species of *Cryptococcus*. Consequently the number of species placed in this genus is small. Ability to assimilate lactose and nitrate are the main characteristics used in the key to the species. It may be that the use of only one carbon compound of significant value for species differentiation has led to a marked oversimplification of the genus. This also holds for *Rhodotorula* and *Trichosporon*. As an example, Phaff, Mrak & Williams (20) stated that certain distinct biochemical differences exist in carbon assimilation reactions between authentic strains of the *Rhodotorula glutinis* group. They believe that a restudy of the genus is needed using a much larger number of carbon compounds, along the lines suggested by Wickerham & Burton (32). An extensive survey of additional carbon compounds should be made to find compounds of particular value for study of species in *Rhodotorula*, *Cryptococcus*, and *Trichosporon*. Acceptable compounds would be assimilated by some species and not by others, and they should yield a minimum of variable, weak, or latent reactions.

Before true relationships among the nonascosporogenous yeasts can become apparent, it is the reviewer's opinion that *Candida*, *Trichosporon*, and *Rhodotorula* must be separated into phylogenetic lines, and that the relationship of these lines to groups outside and inside the area covered by the term "yeasts" must be revealed. In this difficult process much attention will have to be focused on physiology and sexuality. One physiological clue which the reviewer believes may be an important key to the problem is the rather recently discovered ability to produce starch. The relationship of the starch producers in *Cryptococcus*, *Candida*, *Trichosporon*, and *Rhodotorula* should be investigated. There appears some possibility that more than one phylogenetic line is involved. It is now almost pure folly to speculate on related forms outside the yeasts, but it may be worth while to point out one of the less apparent possibilities: Some species of *Taphrina* produce starch, generally in small amounts, and multispored asci; and the writer has found that the multispored yeasts placed in *Lipomyces* also produce starch in shake cultures in large or small amounts. *Lipomyces*, *Cryptococcus*, and *Taphrina* have in common many physiological and morphological characteristics. Is it possible that species of *Lipomyces* are *Taphrina* which do not require a host for sporulation?

An evaluation of starch synthesis as a generic characteristic should be made. It is already indicated by the report of Aschner & Curry (1) on starch production by *Trichosporon* that in closely related species some do and some do not synthesize starch. Starch production may serve better as a guide to the type of yeasts occurring in a phylogenetic line than as a principal characteristic by which genera could be defined.

A study of optimum conditions for starch synthesis is needed,[2] as is a

[2] Since the procedures used at the Northern Regional Laboratory have shown positive results for certain species, and as procedures used at other laboratories have shown negative results for these species, brief mention of our procedures may serve as a point of departure for more critical studies.

Routine determination of starch production is made by adding one or two drops

study of the stability of this characteristic during continuous cultivation of starch-producing species in the laboratory.

The separation of filamentous from nonfilamentous genera is now, as in the past, on different bases for the ascosporogenous and nonascosporogenous yeasts. However, the divergence has been accentuated by Lodder & Kreger-van Rij (17). In the sporogenous yeasts, the genus *Endomycopsis* contains some species which produce an abundance of septate hyphae, while other species form limited amounts. The "nonfilamentous" genera, such as *Saccharomyces* and *Hansenula*, contain some species which produce hyphae with blastospores and some species which form no hyphae at all. In the nonascosporogenous yeasts, species of the "filamentous" genus *Candida* formerly were required to produce pseudohyphae or true hyphae with blastospores, but now need form only groups of elongated cells. This broadening of the genus concept for *Candida* is the reason for transferring to it *Torulopsis utilis* and the entire genus *Mycoderma* Persoon emend. Leberle as a single species, *Candida mycoderma*. Incidentally, while discussing *Mycoderma*, it is of interest to compare the reduction of this genus to a single species with Ciferri & Redaelli's genus of seven comparable yeasts (4) which they place as species under the new genus name *Mycokluyveria*. The reviewer believes both extremes have been reached and that the pendulum probably will swing to a point closer to the concept of the Dutch than that of the Italian workers.

Connell & Skinner (5) reported the ability of yeasts to become filamentous when kept on a modified Sabouraud agar. A large number of yeasts was isolated from skin in 1947 and tested for their ability to produce filamentous growths in corn meal agar scratch plates and gelatin stabs. They were tested twice by both procedures. One hundred thirty-one nonascosporogenous cultures that produced no filaments by either procedure were kept two or three years on Sabouraud slants, then tested again. Seventeen produced filaments by both procedures, and no reversions to their previously

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of weak Lugol's iodine solution to the cultures comprising the carbon assimilation tests when they are three weeks old and are about to be discarded. Positive tests range from black to purple, to blue, and even to green. Where a very weak color is obtained, or when a yeast, because of its taxonomic position might be expected to produce a positive test but does not do so in the routine tests, a flask culture is prepared. The medium used for the carbon assimilation tests (31) is used, plus 3 per cent glucose. The flask is inoculated and shaken vigorously for one to two weeks at 25°C. A few cc. of the culture is placed in a test tube and a drop of the weak iodine solution added. The culture may then be centrifuged and in those strains where the starch is present in the cells and not in the culture medium, the color is concentrated in the sediment. Occasionally a yeast which gives a negative or very weak reaction in cultures of the carbon assimilation tests will give a strongly positive reaction when grown in shaken culture.

Goyette (10) found shaking to favor starch production, but unfortunately he centrifuged his cultures and discarded the cells. Cultures which produce weak reactions often retain all or nearly all of the starch in the cells.

nonfilamentous nature were observed in subsequent tests. Thus, organisms that were originally nonfilamentous, imperfect yeasts of the genus *Torulopsis* had changed to become morphologically identical with yeasts of the genus *Candida*. Connell & Skinner believe both *Candida* and *Cryptococcus* (see following paragraphs) should be maintained for the present, but they believe it should be recognized that cultures of *Cryptococcus* may frequently degenerate into *Candida*.

It is regrettable that Connell & Skinner did not identify the 17 strains that became filamentous with definite species in *Candida* and *Torulopsis*, not only for the sake of further arguments, but also to enlighten the field worker who merely wants to identify his isolates. The authors did, however, cite *Candida pulcherrima* (Lindner) Windisch as a specific example of a yeast which might be placed in either genus according to its normal or "dissociated," "rough" form.

The paper by Skinner (27) on *Cryptococcus* versus *Torulopsis* is recommended reading; it illustrates the depressingly involved confusion which originates in vague old definitions, and which accumulates through the years as attempts are made to redefine the original name or to substitute new names carrying more accurate, but personally variable, descriptions. Skinner concluded that *Cryptococcus* is the valid name and consequently placed under it 14 species previously placed in the genus *Torulopsis*. He expressed the belief that if in the future the genus were divided so that nonfermentative species were placed under the name *Cryptococcus*, the medical profession would be pleased; and if the fermentative species were placed under the name *Torulopsis*, the general yeast taxonomist would be content. As had been noted, Lodder & Kreger-van Rij have followed Skinner's suggestion for dividing the genus, but they divide it on the ability to synthesize starch, not on the ability to produce a gaseous fermentation.

Two yeasts now placed in *Candida* merit specific consideration. Windisch (36) has described, and Roberts (21) has abetted his interpretation of an unusual mode of ascospore formation in *Candida pulcherrima*. Their negligence in failing to stain the presumed ascospores with a spore stain has been criticized by Langeron & Luteraan (12). Most ascospores are acid fast, and a positive stain would have greatly strengthened the contention of both Windisch and Roberts. Lodder & Kreger-van Rij do not believe that the bodies described as ascospores by Windisch in *C. pulcherrima* and *Candida tropicalis* are ascospores; neither do Connell & Skinner (5) nor Wickerham. The latter has repeatedly observed the structures shown in the photographs accompanying Roberts' paper (21). They result from the partial shedding of the outer wall of "pulcherrima cells." Usually one bud, sometimes more, forms on the surface of the inner membrane; and as the bud grows it separates the two walls, the outer forming the lifeless-appearing "ascus" shown in the photographs in Roberts' paper.

Lodder & Kreger-van Rij, in extending the findings of Mrak & McClung (19), found that *Schizoblastosporion starkeyi-henricii* Ciferri, the only species in the genus, no longer buds on a broad base and is very similar to *Candida*

krusei (Cast.) Berkhout. They no longer consider *Schizoblastosporion* to be a valid genus.

Here is a lesson, to wit: On rare occasions a yeast freshly isolated from nature may bud on a broad base, but after some time on artificial media it may no longer show this characteristic. Unless an isolate definitely can be placed in *Saccharomycodes*, *Nadsonia*, *Hanseniaspora*, or *Kloeckera*, the taxonomic significance of broad channels between the mother cells and their daughters should be regarded with suspicion. Some years ago the writer received a culture labeled *Saccharomycodes mestris* (18). On observation it appeared to be an exciting new species of *Saccharomycodes* which conjugated at the end of the vegetative phase, just before sporulation occurred. Recently, on reading Lodder & Kreger-van Rij's manuscript, it was found they had made this name synonymous with *Saccharomyces* (*Zygosaccharomyces*) *acidifaciens*. To the reviewer's surprise, on again observing the strain, its ability to bud on a broad base was no longer apparent, nor was its appearance either unusual or exciting.

There is a possibility that the taxonomic value of the ability to produce carotenoid pigment has been exaggerated. It may be that two major phylogenetic lines meet within the genus *Rhodotorula* and are being confused because both produce carotenoids. Species of one of the postulated lines produce starch and show kinship to *Cryptococcus*. If Lodder & Kreger-van Rij's intimation that some species in the Endomycetaceae may produce carotenoids is correct, the value of this characteristic for generic differentiation is indeed in need of deflation.

Brettanomyces is a genus of nonascosporogenous yeasts, the limits of which are debated by various workers. As recognized by Lodder & Kreger-van Rij, it consists of four species of yeasts which are found almost exclusively in the secondary fermentation of certain beers, particularly lambic beer, a beverage which owes its strongly acidic and distinctive flavor to the long, slow fermentation by species of *Brettanomyces*. The primary characteristics of this genus, according to the Dutch taxonomists, are the ogival shape of some of the cells; strong acid production under aerobic conditions; the acid leading to the early death of the cells; and the ability to assimilate nitrate. Secondary characteristics are the usual development of a primitive pseudomycelium, rarely with blastospores attached to it; the development of a special aroma; slow growth and development of a brownish color in the colonies; and both oxidative and fermentative dissimilation of sugars.

Etchells & Bell (9) have recently described some new species of yeasts which are mainly responsible for that part of the gaseous fermentation of cucumber brines which is caused by yeasts. They may be active at the beginning or toward the middle and end of the cucumber fermentation. One yeast that is active from about the 30th to at least the 100th day of fermentation has been named *Brettanomyces versatilis*. According to Etchells & Bell, it produces ogival cells. The cells are short-lived (four to six weeks), and death is presumably due to accumulation of acids. Acid production is strong under aerobic conditions, but it is less than is produced by the species concerned

in the secondary fermentation of beer. Colonies grown on glucose agar are white at first, then turn light pink to lavender, starting at the center. Nitrate is assimilated. No pseudomycelium is produced.

Lodder & Kreger-van Rij believe Etchells & Bell's *B. versatilis* shows hardly any relationship to the species of the genus *Brettanomyces*, and consequently they place it in *Torulopsis*. The Dutch workers found *B. versatilis* could be kept alive by transferring it, under their conditions, every four months in contrast to the maximum of every two months for yeasts of lambic beer. The colonies change color more slowly and to a lesser degree. The growth of *B. versatilis* is more rapid, the aroma is different, and the presence of ogival cells is very doubtful. The acid production from glucose is only slight, according to the Dutch workers.

The writer is inclined to believe that Etchells & Bell are correct in the placement of this species in *Brettanomyces*, providing the genus can be defined sufficiently well to warrant its continuance. Perhaps the characteristic given most weight by Lodder & Kreger-van Rij is the presence of cells with ogival-shaped ends, often defined as the shape of a Gothic arch. These can usually be seen without difficulty in the lambic yeasts, but it is difficult to avoid seeing them in species of other genera. Whether the shapes seen in a given culture are *bona fide* ogival cells is occasionally open to debate, and even the Dutch workers state that the presence of such cells in *B. versatilis* is doubtful, whereas Etchells & Bell state without qualification that they are present. Any characteristic that can be variously interpreted is of doubtful value as the main characteristic of a genus.

The second and third most weighty characteristics in *Brettanomyces* are strong acid production and the ability to produce enough acid to kill the cells rapidly. It may be assumed that some variation will occur in the amount of acid produced by species of this genus. The Dutch authors compared the longevity of Etchells & Bell's species with that of the lambic yeasts, whereas the comparison should also have included yeasts from other nonascosporogenous, nonpigmented genera. Longevity, the rate of growth of colonies, the rapidity with which they turn brownish or lavender, and the aroma, may all be a reflection of the one fact that *B. versatilis* produces less acid than the lambic yeasts. Though it may die somewhat less rapidly than the lambic yeasts, *B. versatilis* is, in the writer's opinion, certainly a short-lived yeast, as the phrase is used in connection with the *Brettanomyces*.[3]

Pseudomycelium is lacking in *B. versatilis*, and this reviewer has looked vainly for it in *B. claussenii* Custers, a species recognized by the Dutch workers. Thus filament formation can hardly be used to exclude a species from this genus. Incidentally, *B. versatilis* and *B. claussenii* are the only two species of yeasts which are exceptions to the rule that a yeast which ferments

[3] The reviewer hesitates to use the term "short-lived," for he has found that old cultures of *Brettanomyces* may exist in a limbo between life and death. Such moribund cells will not grow when streaked lightly on ordinary agar media. However, if certain actively growing yeasts are spotted onto the agar, satellite colonies of the *Brettanomyces* will appear around the developing colony.

lactose does not ferment maltose. *Brettanomyces versatilis* assimilates nitrate, as does *Brettanomyces* by the presently accepted generic definition.

Etchells & Bell (9) described another new species, *Brettanomyces spheri-cus*, which Lodder & Kreger-van Rij likewise placed in *Torulopsis*. Some of the same disagreements between the two groups of workers hold for this species as for *B. versatilis*.

Verona & Florenzano (30) described *Brettanomyces italicus* as a slow-growing species which formed small colonies that were at first white and later brownish, especially at the center. Cells were short-lived, and some were ogival in shape. A rudimentary pseudomycelium was formed. Nitrate was reported to be assimilated, but Lodder & Kreger-van Rij could not confirm this; neither could they confirm the presence of ogival cells. The Dutch authors classify this yeast as a questionable strain of *Torulopsis bacillaris*.

Species of *Brettanomyces* and other yeasts which may be related to them have long intrigued the writer. To him they appear to belong to a phylogenetic line that may be quite different from any of the other lines of non-ascosporogenous yeasts. They are usually found in commercial fermentations of marked acidity, are strongly resistant to high osmotic pressure, are generally difficult to maintain as agar cultures though they can be kept easily by lyophilization, and they do not store reserves of vitamins in the cells. The reviewer believes both filamentous and nonfilamentous, and nitrate-positive and nitrate-negative yeasts may belong to this group, and there are species of intermediate degree in both categories. The range would cover such nonfilamentous and nitrate-negative species as *Torulopsis bacillaris* (Kroemer et Krumbholz) Lodder and *Torulopsis stellata* (Kroemer et Krumbholz) Lodder; the nitrate-positive nonfilamentous yeasts, represented by *Torulopsis lactis-condensi* (Hammer) Lodder et Kreger-van Rij, *Torulopsis etchellsii* Lodder et Kreger-van Rij, and *Torulopsis anomala* Lodder et Kreger-van Rij of the Dutch system; and include as well *B. versatilis* of Etchells & Bell, and the lambic beer yeasts. Fundamental work must be done before this suggested relationship could appear convincing.

An over-all evaluation of the book by Lodder & Kreger-van Rij must be given in concluding this report of their work. It is natural that this reviewer has given little space to advancements made by the Dutch authors with which he concurs, whereas he has discussed at length areas of work where he believes further progress may be made. It is evident that his long-range suggestions will be difficult to consummate.

The new book by the Dutch authorites is expected to provide stimulus to research in yeast taxonomy, particularly in English-speaking nations. Its value as a tool for the field worker who wants to identify his isolates can hardly be overestimated. A substantial reduction in the number of recognized species and varieties makes identification considerably easier than it has been in the past. Possibly the authors' treatment of *Zygosaccharomyces* and *Debaryomyces* may rid yeast taxonomists of the habit of unduly splitting species in these genera. Their treatment of other genera may inhibit the incipient formation of this same habit with *Hanseniaspora*, *Nematospora*, and

Nadsonia. The excellent bibliography, the stated source of cultures, and the availability of the cultures from the Centraalbureau collection constitute a very solid foundation for the projects of zymologists working in many different fields.

Writing so comprehensive a book in the English language is a personal achievement of which Dr. Lodder and Mrs. Kreger-van Rij may be justly proud, and for which we can be very thankful.

So far as this reviewer is aware, in the United States the only textbook devoted entirely to taxonomy of yeasts is that by Mrak, Phaff, & Stadtman entitled, *Taxonomy and Morphology of Yeasts*. It is composed of some 300 mimeographed pages, this mode of printing permitting insertion of additional sheets as new information becomes available. In its present state, the text contains chapters on taxonomy and morphology, ecology and distribution, keys to the orders, families, and genera, and dichotomous keys to the species according to Stelling-Dekker (29), Lodder (15), Diddens & Lodder (7), and Wickerham (31). Complete descriptions of the species are not included, for inclusion of such voluminous material would be next to impossible. The remaining chapters cover new species of yeasts described in the more recent literature, laboratory directions, and miscellany, this last chapter dealing mainly with metabolism. Bibliographies direct students to pertinent publications, most of which are relatively recent. The book is well illustrated. It is bound in a paper cover. Dr. Mrak has informed the reviewer that he does not consider their text a book, nor does he desire much distribution of it. He further stated that it "is merely a compilation of material that we got together in a hurry for our class."

The first chapter is of special interest to this review because it deals with phylogeny, along the same lines as reported by Mrak & Phaff in 1948. These authors believe the families Dipodascacae and Taphrinaceae as well as Endomycetaceae, should be considered as yeasts. Of the more closely allied genera, they would include *Ashbya* and *Eremothecium*. They believe *Pullaria pullulans* (de Bary et Löw) Berkhout should be included in the yeasts, since strains which fail to form the typical brown to black spores would be indistinguishable from *Trichosporon pullulans*. *Geotrichum* would be included, as it seems quite evident to the investigators at the University of California that this genus should contain imperfect forms comparable to the subfamily Endomycoideae of Stelling-Dekker. It is of interest to recall, however, that previously in this review it was reported that Lodder & Kreger-van Rij (17), rather than adding *Geotrichum*, have now excluded from the yeasts the only genus of ascosporogenous species directly analogous to *Geotrichum*, namely, *Endomyces*.

It is evident that zymologists in the future probably will disagree increasingly on which fungi are to be included in their territory, but they show a general tendency to take over more genera and families. It is hoped that a study of the occupied areas will progress to a point where separation into major groups is clearly apparent, and that those groups which do not conform to the area as a whole will be placed in more natural alliances.

Leupold (13) has reported the presence of heterothallic, homothallic, and nonsporogenous cells within a single culture of *Schizosaccharomyces pombe* Lindner. Wickerham & Burton (33) have briefly reported on some yeasts which exist in the vegetative state entirely or predominantly as haploid cells, and are heterothallic. Nearly all strains of the species which they cited by name may be depended upon to form ascospores when cells of the opposite mating types are thoroughly mixed together on a slant of sporulation medium, then spread out over the surface of the slant, and incubated at 25°C. Such species are *Zygosaccharomyces lactis* Dombrowski [separate mating types are known as *Torulopsis sphaerica* (Hammer et Cordes) Lodder]; *Endomycopsis ohmeri*, a species recently described by Etchells & Bell (8); and a yeast of which Berkhout (3) has given us the best description to date under the name *Candida chodati* Nechitsch.

Wickerham & Burton mentioned, but did not discuss the fact that they had isolated several heterothallic species from nature which exist exclusively as haploids in the vegetative state. One of these species belonging in *Hansenula* is very frequently isolated, and the isolates have never been observed to produce ascospores when inoculated separately on sporulation medium. However, two isolates from the same geographic area mate when mixed and produce spores up to 50 per cent of the total cell count. When these two mating types are paired with other isolates, one occasionally produces up to 1 per cent of spores; the other has never mated with other strains of this same species. Thus far, in this species of which we have already studied over 60 strains, only this one set of mating types has been found which sporulates well. If the same factors which limit the function of mating types in this species are present in less commonly isolated yeasts, the chances of obtaining the perfect forms of such yeasts are, with our present state of knowledge, indeed slim. It is certainly anticipated, however, that the number of yeasts known to be heterothallic will gradually increase to a large number as compared with the few known at present.

Schultz & Pomper (26), Schultz, McManus, & Pomper (25), and Schultz & McManus (24) have reported some interesting results on the ability of amino acids to serve as sources of nitrogen and carbon for the growth of yeasts, and of various sulfur compounds to serve as sources of this element. It is possible that their work may serve as the basis for additional biochemical tests in the classification of certain genera of yeasts. Evaluation would involve the study of many strains in each of many species to determine the amount of variation to be encountered.

LITERATURE CITED

1. Aschner, M., and Cury, A., *J. Bact.*, **62**, 350–51 (1951)
2. Bedford, C. L., *Mycologia*, **34**, 628–49 (1942)
3. Berkhout, M., *De Schimmelgeschlachten Monilia, Oidium, Oospora en Torula* (Doctoral thesis, Univ. of Utrecht, 1923)
4. Ciferri, R., and Redaelli, P., *Mycopathologia*, **4**, 54–59 (1947)
5. Connell, G. H., and Skinner, C. E., *Mycopathologia* (In press)

6. Derx, H. G., *Ann. Mycol.*, **28**, 1–23 (1930)
7. Diddens, H. A., and Lodder, J., *Die Anaskosporogenen Hefen, Zweite Hälfte* (N. V. Noord-Hollandsche Uitgevers Maatschappij, Amsterdam, Holland, 509 pp., 1942)
8. Etchells, J. L., and Bell, T. A., *Food Technol.*, **4**, 77–83 (1950)
9. Etchells, J. L., and Bell, T. A., *Farlowia*, **4**, 87–112 (1950)
10. Goyette, L. E., *Trans. Kansas Acad. Sci.*, **54**, 28–35 (1951)
11. Holst, E., *J. Agr. Research*, **53**, 513–18 (1936)
12. Langeron, M., and Luteraan, P.-J., *Ann. parasitol. humaine et comparée*, **22**, 254–75 (1947)
13. Leupold, U., *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **24**, 381–480 (1950)
14. Lindegren, C. C., and Lindegren, G., *Ann. Missouri Botan. Garden*, **30**, 453–68 (1943)
15. Lodder, J., *Die Anaskosporogenen Hefen, Erste Hälfte* (Koninklijke Akademie van Wetenschappen, Amsterdam, Holland, 256 pp., 1934)
16. Lodder, J., *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **12**, 273–80 (1947)
17. Lodder, J., and Kreger-van Rij, N. J. W., *The Yeasts. A Taxonomic Study* (North-Holland Publishing Company, Amsterdam, Holland, 650 pp., 1952)
18. Marcilla, J., and Feduchy, E., *Centros de estudios generales y de ampelografía y viticulture*, **29**, 1–40 (1943)
19. Mrak, E., and McClung, L., *J. Bact.*, **40**, 395–407 (1940)
20. Phaff, H. J., Mrak, E. M., and Williams, O. B., *Mycologia* (In press)
21. Roberts, C., *Farlowia*, **2**, 345–83 (1946)
22. Routien, J. B., *Mycologia*, **41**, 183–85 (1949)
24. Schultz, A. S., and McManus, D., *Arch. Biochem.*, **25**, 401–9 (1950)
25. Schultz, A. S., McManus, D., and Pomper, S., *Arch. Biochem.*, **22**, 412–19 (1949)
26. Schultz, A. S., and Pomper, S., *Arch. Biochem.*, **19**, 184–92 (1948)
27. Skinner, C. E., *Am. Midland Naturalist*, **43**, 242–50 (1950)
28. Starkey, R. L., *J. Bact.*, **51**, 33–50 (1946)
29. Stelling-Dekker, N. M., *Die Sporogenen Hefen* (Koninklijke Akademie van Wetenschappen, Amsterdam, Holland, 547 pp., 1931)
30. Verona, O., and Florenzano, G., *Atti accad. naz. Lincei, Rend. classe sci., fis., mat. e nat.*, **3**, 383–87 (1947)
31. Wickerham, L. J., *U. S. Dept. Agr. Tech. Bull., No. 1029* (1951)
32. Wickerham, L. J., and Burton, K. A., *J. Bact.*, **56**, 363–71 (1948)
33. Wickerham, L. J., and Burton, K. A., *J. Bact.*, **63**, 449–51 (1952)
34. Wickerham, L. J., and Duprat, E., *J. Bact.*, **50**, 597–607 (1945)
35. Wiles, A. E., *J. Inst. Brewing*, **56**, 183–93 (1950)
36. Windisch, S., *Arch. Mikrobiol.*, **11**, 368–90 (1940)
37. Winge, O., and Laustsen, O., *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **22**, 99–116 (1937); **22**, 235–44, (1938); **22**, 337–53 (1939)
38. Yukawa, M., and Maki, T., *Bul. Sci. Fakultat. Terkult., Kjusu Imp. Univ. Fukuoka Japan*, **4**, 218–226 (1931)

BACTERIAL VIRUSES[1]

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Within the past several years several reviews have appeared on bacterial viruses. For this earlier work, the reader is referred to the reviews of Cohen (1), Anderson (2, 3) and Fong (4). Delbrück has edited an excellent review summarizing the results on the *Escherichia coli* B system (5). The recent article by Luria (6) should also be consulted for those wishing a stimulating discussion of the growth of phage in the *E. coli* B system. The physical properties of phages have been summarized by Putnam (7).

The present article should not be considered a complete review of the literature since Anderson's paper appeared in this series in 1950 (3). Although most of the papers referred to in the present article have been published since 1950, this paper is more a summation of the present status of the phage problem.

Particular attention has been paid to the biochemical aspects of phage reproduction in this review. This was done, since at the present stage, the problem of phage synthesis is a biochemical one. This is not to say that the biological and genetic approach which has given us so much fundamental knowledge will not continue to do so. However, the mechanism of virus synthesis must primarily come from biochemical studies aided by biological, genetic, and immunological investigations.

The review has been divided into six parts: (*a*) the adsorption and invasion of host cells, (*b*) the synthesis of virus material, (*c*) the release of the virus particles, (*d*) lysogenesis, (*e*) the relationship between bacterial viruses and animal viruses, and (*f*) the summary.

The Problem

The main problem in phage synthesis is to understand how a part of the infecting phage can control the bacterial enzymes in such a manner that the energy and metabolic systems of the infected cell are directed toward the replication of the whole infecting particle. The synthesis of phage may be divided into two parts: (*a*) the formation of nonspecific phage material which takes place through the normal cellular metabolic pathways, and (*b*) the synthesis of specific phage material. The mechanism for this second step is not understood.

Adsorption and Invasion

Because of the recent work of Puck and co-workers (8, 9) it now seems clear that the adsorption of the virus to the cell takes place in two steps in

[1] The survey of the literature pertaining to this review was concluded in April, 1952.

the *E. coli* B system. The initial interaction between host cell and virus consists of a very rapid reversible step, whose rate can be controlled by the salt constituents of the medium. It is this first step that requires the cofactor tryptophane. This reversible adsorption is followed by a reaction which leads to an irreversible adsorption of the virus to the host cell and appears to be an enzymatic process. It is this second step which results in the "killing" of the host cell and will be called "invasion."

Important recent experiments by Herriott (10) have shown that the adsorption and "killing" properties of T2r$^+$ phage particles reside exclusively in the ghosts of the virus. These ghosts consist of the deflated head and tail of the particle and contain no DNA (desoxyribonucleic acid). They also contain all the antigenic structure of the phage (11, 12). The DNA released from the head of the phage cannot be adsorbed to susceptible cells (11, 12).

Once the virus is irreversibly adsorbed to the host cell, some marked changes occur in the phage. It has been shown by Doermann (13, 14) and Anderson & Doermann (15) that after adsorption, the infectivity of T4 and T3 particles is lost, and no infective particles can be recovered until about the middle of the latent period. This was the first demonstration that some marked change took place in the phage before it began to reproduce. Chemical studies have also shown that the phage undergoes changes on adsorption. Putnam & Kozloff (16), working with T6r$^+$, observed that when the phage was labelled with P^{32}, the greater part of the isotope, 49 to 63 per cent, was found in the medium as unsedimentable low molecular P. In a continuation of these studies, Kozloff found that 14 per cent of the virus N^{15} was liberated from one infecting T6r$^+$ particle within 10 min. after adsorption (17). He also showed that the secondary infecting particles are broken down to the same extent as the initial particle. This conclusion seemed at variance with the results reported by Lesley, French, Graham, & van Rooyen (18), who found that only 5 per cent of the T2 phage labelled with P^{32} was broken down, using the release of P^{32} from the phage as a measure of virus breakdown. Secondary infecting T2 particles were broken down to the extent of 55 per cent (18). The breakdown of the secondarily invading T2 particles is stimulated by previous infection of the cell with T2, T4, T5, and T6 phages, but not with T1, T3, and T7 (19). The differences in the results of Kozloff (17) and Lesley *et al.* (18) are due to the fact that the former's experiments were carried out in synthetic lactate medium, and the latter's experiment in nutrient broth. In the former medium, the reaction which leads to the breakdown of the secondary infecting phages is not stimulated by primary infection (17).

In one of the most fundamental virus studies that has appeared in some time, Hershey & Chase (12) have found that on adsorption about 80 per cent of the phage protein remains on the bacterial surface, with the DNA passing into the cell. The role, if any, of the other 20 per cent of the phage protein in virus synthesis is not clear. However, it appears quite certain that the principal role of most of the phage protein is to get the virus DNA into the cell.

This was suggested by Northrop previously (20). Once the DNA is inside the cell it seems to form some sort of organized intracellular complex (12). From these experiments (12) one might think that the breakdown of secondary infecting particles referred to above might be due to a stimulation of the bacterial desoxyribonuclease by the primary infecting particle. If the phage is adsorbed to bacterial debris, the virus particle loses about one-half of its DNA (12). The remaining DNA can be digested away with desoxyribonuclease. The DNA in normal phage particles is not acted upon by desoxyribonuclease. Anderson recently reported that T2 may become attached to the host cell by its tail (21). Weidle found that T2 phages in contact with purified cell membranes dissolve these cell structures (5). These facts would tend to indicate that the phage protein makes an attachment and entrance in the cell structure, and that the DNA from the head of the phage then passes into the cell. The mechanism of this phenomenon has been thought of in terms of a hypodermic needle injection of transforming principles (11).

It is during the adsorption and invasion reactions that many of the interference phenomena between bacteriophages are established. It has been known for many years that dissimilar phages will exclude each other (22). For example, T2 will exclude T1, when both are added to the cell at the same time (22). This is the mutual exclusion effect (23). Although T1 does not reproduce in such cells, it lowers the yield of T2. This is known as the depressor effect (23). Hershey, working with T2 and T2r, showed that cells simultaneously infected with these viruses would liberate both types of viruses (24). This meant that the mutual exclusion effect did not hold for similar viruses. This result has been confirmed using pairs of other similar viruses (25, 26, 27). Dulbecco (28) recently observed, however, that the mutual exclusion effect will hold amongst similar viruses if the secondary infecting particle is added several minutes after the primary particle. He found that in nutrient broth $T2r^+$, added one minute before, excludes T2 in 50 per cent of the bacteria, and vice versa. Phage killed by irradiation will also exclude the superinfecting particle (28). Herriott (29) has observed that although $T2r^+$ will exclude the superinfecting T4r phage in nutrient broth, it will not do so in a glucose synthetic medium even when the T4r is added ten min. after $T2r^+$. These experiments are of interest in view of the results referred to above where the primary particle stimulated the cell to break down the secondary infecting particles in nutrient broth but not in a synthetic medium (18). It seems, therefore, that the interference between similar viruses is probably due to the breakdown of the superinfecting particles. The interference between dissimilar viruses such as T1 and T2, however, does not appear due to this mechanism since T2 will exclude T1 when both simultaneously infect a cell, whether the experiment is carried out in nutrient broth or in a glucose synthetic medium (29). The ability to exclude in the T series of viruses resides in the ghosts (29). $T2r^+$ ghosts will exclude T1 and also T4 under the experimental conditions where $T2r^+$ phage will exclude T1 and T4 (29). These results make it certain, therefore, that the interference

phenomena in the T series of bacterial viruses is not in any way related to the multiplication of the virus. Whether the ghost can cause the depressor effect is not known. It would be of very great interest in this system to study nuclear changes of ghost-infected cells. In all previous instances, whenever a virus particle excluded another one, the ability to exclude was destroyed at the same rate as its ability to kill bacteria. Furthermore, the bacterial nucleus was broken down.

In a recent study carried out on the lysogenic *E. coli* strain K12, it was shown that T5 would exclude the virus particle normally liberated by the bacteria even when it was added quite late in the reproductive cycle of the lysogenic phage (30). The results indicate the interference can be established well past the adsorption-invasion stage of the phage reproductive cycle. Similar experiments should be carried out with the T2–T1 exclusion system to determine how long T2 can be added after T1 and still cause interference. This experiment should be correlated with a biochemical analysis in order to determine at what step in the synthesis of the phage interference can or cannot be established.

In all cases studied the resistance of bacteria to phage is due to the failure of such bacteria to adsorb the phage. Such studies do not tell us whether the phage would multiply if it was adsorbed to such cells. The recent experiments of Novick & Szilard (31), however, show that at least in one case, the resistance is due solely to lack of adsorption. They found on mixed infection of *E. coli* B with T2 and T4 some particles were liberated which had the phenotype of T4, but the genotype of T2. Thus, they would multiply on B/2, normally sensitive to T4 and not to T2, and the virus progeny would multiply on B/4 but not on B/2. The host specificity in T2 is controlled by genes occupying at least two different *loci*, at least one of the genes being capable of alternative mutations (32).

The Synthesis of Virus Material

Once the phage invades the cell, certain marked changes occur in the host's metabolism. In *E. coli* B and *Staphylococcus muscae* protein is synthesized from the beginning of the infection (33, 34). The amount of protein formed is always more than that required for the amount of virus. In an *S. muscae*-infected cell there is about twice as much protein formed as is required for virus synthesis (34). There is also more protein formed in the *E. coli* B systems than is necessary for the amount of phage formed (33). Because of technical difficulties, however, there is no really good information available on just how much excess protein is formed in the latter system. The question immediately arises whether all the protein formed is virus protein. Until all this protein can be identified, and the connection between virus protein, nucleotides, and higher molecular weight building blocks for virus DNA synthesis better defined, the relationship between protein and DNA synthesis in infected cells will remain unclear.

The problem of the extra formation of protein could, of course, be an-

swered by using N^{15} together with immunological tests, and see whether any protein but virus protein is formed. Kozloff *et al.* (35), using N^{15}, showed that from 75 to 95 per cent of T6r$^+$ phage protein came from the medium after infection, the remaining 5 to 25 per cent being contributed by the host. The Chicago group found that the debris from a T6r$^+$ lysate obtained by centrifuging the lysate at low speed contained about 20 per cent of the total N^{15} (36). This does not mean that bacterial protein was formed, however, since it could be due, at least in part, to adsorbed N^{15} phage.

A study of the effect of various amino acids, which are constituents of the phage, has been carried out on the *S. muscae* system (37). Under conditions of single infection, the failure to add any one of these amino acids to the medium resulted in the adsorption of the virus particle and the subsequent killing of the cell. One-step growth curves, and protein and DNA determinations indicated that in such cells there was little if any virus material synthesized, and the infective cycle went little beyond the invasive stage. These results are understandable since the synthesis of protein appears to be the first synthetic reaction leading to the formation of the complete particle. When cells in such depleted substrate media were infected with several phage particles, virus was liberated, the yield depending upon the multiplicity of infection (37). Experiments showed quite conclusively that the virus liberated was newly synthesized phage and not that which was added. A similar effect of multiple infection increasing the phage yield was recently reported by Barry & Goebel (38) with Phase II *Shigella sonnei* infected with T4.

In *E. coli* B, the uptake of tryptophane from the medium was faster with cells infected with T2 than with normal cells (39). The uptake in the presence of T4 was slower than with T2 but faster than T6 (39).

A great deal more emphasis is certainly warranted on protein synthesis by phage-infected cells. One way to attack this problem would be to use an *E. coli* B tryptophane requiring mutant and a T virus containing tryptophane. Radioactive tryptophane could be added to the medium and its metabolic pathway followed in normal and infected cells.

The nucleic acid composition of an *E. coli* B virus is much different from that found in the host. T2r$^+$, T2, T4r, T4r$^+$, T6r, and T6r$^+$ seem to consist exclusively of DNA (99 per cent) (40). Herriott (41) has found similar values with T2r$^+$. Furthermore, T2r has been found to contain little, if any, cytosine (42). Similar findings have more recently been obtained with T6r and T6r$^+$ where there is markedly less cystosine than thymine, a situation not found in the host (43). T5, on the other hand, is reported to contain a fair amount of cytosine (44).

Much work has been done on the nucleic acid metabolism of infected cells. Cohen has made notable contributions in this field (45). On infection, the nucleic acid metabolism of *E. coli* B infected cells is inhibited for about 10 min. (45). However, there is an increase in the ultraviolet reading at 2600 Å beginning at about 7 min., indicating a synthesis of purine and pyrimidine containing materials which go to form virus DNA (46). Once the

DNA synthesis begins, it increases arithmetically and at four times the rate found in uninfected cells (45). There is no increase or turnover in RNA (ribonucleic acid) in contrast to uninfected cells which contain three times as much RNA as DNA (45). The amount of DNA formed is about equal to the combined DNA and RNA synthesis in uninfected cells (45). It appears as if all the low molecular weight building blocks for RNA are shunted toward virus DNA. The formation of virus DNA precedes the completion of the infective particle by a few minutes (45). The amount of DNA formed is about that required for the amount of virus synthesized (45). Similar findings have been made with *S. muscae*, although in this case RNA appears to be synthesized as well as virus DNA (47). Even if one assumed the amount of RNA found in *S, muscae* phage purified by differential centrifugation to be part of the virus, for which there is no rigorous evidence, there is still much more RNA synthesized than is necessary for the amount of virus formed (47)[2]. More work is needed to clarify the relationship between RNA synthesis and virus reproduction in this system.

The amount of DNA and protein formed in the *S. muscae* system depends upon the phage yield and also on the rate of synthesis of these two substances in the host cell at the time of infection (34). This is further evidence that the virus mobilizes the cell enzymes present at the time of infection for the synthesis of the nonspecific building blocks necessary for virus reproduction. In this connection, attempts to demonstrate enzyme activity in purified preparations of T2 (48) and *S. muscae* phage (49) were unsuccessful. In the latter studies phage preparations containing about 5×10^{12} particles were employed. Tests for all the reactions in glycolysis and those in the Krebs cycle were negative. There was no oxidation of gluconic acid, the 21 common amino acids, or of any of the fatty acids that were tried. All the essential known cofactors were added in these tests plus extracts of boiled cells. The methods used were sensitive enough to detect the enzymes in the phage if they had been present in one-tenth the amount found in the host cells. Furthermore, in view of the fact that the material released from the head of the phages seems to be pure DNA (11), the enzymes would have to be associated with the phage ghosts. It is unlikely, therefore, that permeability plays too large a role in the negative results.

Pardee (50) has shown that when *E. coli* B is infected with T3 there is an increase in the adenosinetriphosphatase activity of the cells. Whether this means that there is an increase in the amount of the enzyme on infection is far from clear, however, as many other alternatives would have to be ruled out.

About 70 to 80 per cent of the phosphorus appearing in the virus DNA in $T2r^+$ comes from the medium after infection (1). Similar results were

[2] Although there is always more RNA formed than could possibly be accounted for in the virus, the amount of RNA synthesized depends upon the phage yield; the more phage reproduced, the more RNA formed.

obtained with T6r$^+$ (51). With T7, however, most if not all of the phosphorus is derived from the host (52). There was no net increase of DNA in this system. Furthermore, the data show that the phosphorus comes from the bacterial DNA and not from the bacterial acid soluble phosphorus present at infection. Labaw (53) using T1, T2r$^+$, T2r, T3, T5, and T7 has made a further comparative study to determine how much of the virus phosphorus comes from the medium after infection and how much comes from the bacterial host. He found that the virus phosphorus having its origin in the bacteria before infection is constant. However, since the total phosphorus content per lytic unit is approximately proportional to the size of the phage, the amount of phosphorus found in the virus which is present in the host at the time of infection varies from 20 to 80 per cent of the total phosphorus present in the different bacterial viruses. In a further study of this problem, Labaw (54) observed that with T5 most of the bacterial phosphorus found in the phage does not come from the DNA of the host but presumably comes from smaller molecular weight substrates. This result is different from that which has been obtained with the other T viruses studied, where the bacterial DNA was found to be the chief contributor of bacterial phosphorus. However, since these latter studies showed that the bacterial DNA was first broken down to nucleosides and nucleotides before being used for virus synthesis, there does not appear to be any real discrepancy between the results found with T5 and the other T phages.

Similar conclusions regarding the contributions from the bacterial host DNA to the virus progeny have also come using cells labelled with the bases of nucleic acid. Koch (55) showed that there was a transfer of bacterial purines to the phage progeny. It was suggested, on the basis of experiments which showed that the amount of bacterial nucleic acid found in the progeny was independent of the time of lysis and the yield, that each virus particle received the same proportion of host DNA (56). Weed & Cohen (43) found, however, that the last three-quarters of the virus produced within the infected cell received none of the host pyrimidines. These authors also presented evidence that units of bacterial DNA larger than nucleosides or nucleotides are not involved in the formation of virus DNA.

Not only is bacterial DNA contributed for the reproduction of new phage, but about one-third of the phosphorus of the infecting particle is contributed to its progeny (16). This result has been confirmed (59). In studying this phenomenon, Hershey and co-workers (57) showed that the phosphorus contributed by the infecting particles definitely does not all appear in one phage progeny. In the light of genetic recombination, where there is a redistribution of functionally conserved structures during virus growth (58), Hershey and co-workers (57) suggested that the parent phosphorus contribution may consist of essential structures which are conserved during phage multiplication. The experiments of Maaløe & Watson show that this cannot be the case (59). T2r$^+$ phage labelled with P^{32} was carried through two cycles of reproduction. In both cycles, the phage progeny received about 30 per

cent of the P^{32} of the infecting phage particles. Thus, it is clear that the phosphorus transferred from the parents to the progeny is not located in specific parts of the DNA of some or all of the progeny. A similar conclusion was reached by Kozloff (60). His results showed that the contribution of the infecting $T6r^+$ particles to their progeny is mainly of a nonspecific nature.

All these results therefore make it quite clear that there is no specific substrate contribution by either the infecting particles or the host to the phage progeny in so far as virus protein and DNA are concerned.

Recent experiments by Cohen (45) have indicated that one major shunt in the nucleic system in the *E. coli* B-phage system occurs at the glucose-6-phosphate stage. Enzyme studies have revealed that the oxidation of glucose-6-phosphate, leading eventually to the formation of ribose-5-phosphate, is one of the paths that could be used for the synthesis of the ribose phosphate necessary for RNA synthesis (45). Phage infection has been found to inhibit this pathway in *E. coli* B. The substrates normally used for ribose-6-phosphate may then be shunted into the Embden-Meyerhof series of reactions leading to the formation of desoxypentose phosphate by Racker's (61) reaction, i.e., phosphoglyceraldehyde plus acetaldehyde→desoxypentose phosphate. It remains to be seen whether the inhibition of the formation of ribose-5-phosphate through the oxidation of glucose-6-phosphate can account for the total inhibition of RNA synthesis in *E. coli* B infected cells.

The above experiments further show that on phage infection there is an inhibition at the enzymatic level. It is difficult to fit the above findings in with Luria's view (62) that the failure of phage infected cells to carry out the synthesis of normal cellular components is due to the destruction of the bacterial nucleus which occurs on phage infection. Even if the bacterial nucleus is destroyed, the enzymes for the oxidation of glucose-6-phosphate are still present in the cell. It would be of interest to make cell free extracts of the infected bacteria and test them for the above series of reactions, and also to add phage ghosts and virus DNA, obtained from the head of the phage to bacterial extracts capable of carrying out the above reactions. The inhibition of RNA synthesis in *E. coli* B infected cells, furthermore, does not depend upon virus multiplication, since Herriott (10) has observed it with phage "ghosts." More in keeping with Luria's above hypothesis is the finding that cells which are treated with ultraviolet irradiation (63) or mustard gas (64) can form virus, although they can no longer synthesize bacterial DNA. These results indicate that the virus DNA plays a direct part in synthesizing more virus DNA from the low-molecular weight nonspecific building blocks. The bacterial DNA probably acts in a similar manner in the normal cell to form its nucleic acid. Evidence for this is found in the observations that treatment of *E. coli* B with the above agents and ultraviolet irradiated phage (65) all probably affect the bacterial DNA and the ability of the cell to form DNA, but do not inhibit the reactions which synthesize the lower molecular weight building blocks necessary for DNA synthesis.

Virus Liberation

In most instances liberation of the bacterial virus is directly correlated with cellular lysis (5). However, in the *S. muscae* system, under certain conditions, one strain liberates virus without cellular lysis as determined by turbidimetric and, more critically, microscopic examination (66). However, such cells do eventually lyse. In all other strains of *S. muscae* virus liberation occurs simultaneously with cellular lysis under all conditions studied (66). The phage liberated from the former and latter strains of *S. muscae* cannot be distinguished by plaque formation, nutrient requirements, or by serological tests. It therefore appears that the "lysis difference" between the strains resides in the host cell rather than in the phage that is formed. Northrop (67) recently presented evidence that under certain conditions a susceptible strain of *Bacillus megatherium* may release phage without lysis.

Two kinds of lysis have been distinguished in phage infection. One type, known as "lysis from without," occurs when a large number, 100 or more, particles are adsorbed by the cell (5). Lysis comes quickly and no phage is released. Lysis from without is also brought about when cells are infected with a few phage particles in the presence of iodoacetate, cyanide, or dinitrophenol (1). However, the latter result is found only when *E. coli* B is infected with T2r$^+$ but not with T2r (68). When the above three metabolic inhibitors are added to cells infected with T2r, lysis is inhibited rather than accelerated (68). Similar results are observed with the *S. muscae* system (66). The explanation of Cohen (1) that lysis from without in the presence of a few phage particles and a metabolic inhibitor results from "the host's energy supply being uncontrollable," therefore, does not hold for all types of phage systems. The lysis that takes place when cells are infected in the absence of a utilizable carbohydrate source (68) or with phage ghosts (10) also appears to be due to "lysis from without." This type of lysis may occur without the breakdown of the cell nuclei (5). "Nonkilling" x-ray inactivated phage can also bring it about (69). These experiments indicate that "lysis from without" need not be due to the invasion of the cell and can occur independently of the synthesis of virus specific materials.

The mechanism resulting in the lysis of cells infected with a few phage particles is not known. It was shown several years ago that cells infected with a few particles all lyse at approximately the same time irrespective of whether they yield only a few viruses or several hundred phages (70). "Normal" infection seems to initiate a series of reactions which lead to cellular lysis at a definite time interval. The time at which the cells lyse depends upon the virus used to infect the host (5). Evidence has been accumulated that the loss of RNA from the infected cell may be one of the important reactions leading to lysis (47, 66). *E. coli* B cells infected with T2r$^+$ were also shown to lose RNA well before the time lysis begins (71). It would be of interest to correlate the release of RNA from *E. coli* B cells infected with all the T phages in relation to the time the cells lyse.

Lysogenesis

Within the last few years there has been a renewed interest in lysogenic strains of bacteria. In such strains, each bacterial cell is capable of carrying the potentiality for synthesizing virus without any phage having to be added. At the same time, the lysogenic cell is able to grow and divide.

Lwoff & Gutman have reported that under their experimental conditions phage liberation in *Bacillus megatherium*, strain 899, is correlated with bacterial lysis (72). This result has been confirmed by Clarke & Cowles (73) and by Bertani (74) working with *B. megatherium* 899, and *E. coli*, strain "Li." Bertani made the interesting observation that although strain "Li" liberates three types of phage, phage bursts are always homogenous as to the phage type (74). Lwoff & Gutman (72) also noted that lysis of isolated single cells of strain 899 does not occur under conditions where lysis of several cells does take place, a result which has been confirmed by Clarke & Cowles (73).

Northrop (20) on the other hand, working with lysogenic *B. megatherium* strain 899a, has reported that the bacteria under certain experimental conditions may form and release phage and still grow and divide. The average phage yield in these experiments is only about two particles. His case would be much stronger if some way could be found to determine the phage yield from single cells. This would rule out the possibility that the phage was coming from a few lysing cells which could not be detected. He also made the interesting observation that at the time the cells begin to form phage in yeast extract medium they will not form colonies on yeast extract agar; i.e., they are no longer viable. However, no evidence of lysis is detected in the mass culture. As soon as the cells stop synthesizing phage, the cells are again viable when plated on the latter medium. This result does not appear due to selection or spontaneous mutation. The results indicate that when the phage titer begins to increase in the mass culture, all the cells in the culture appear much more sensitive to cultural conditions (20).

The most important aspect of lysogenicity is centered around the problem of the factors which determine whether the cell will or will not synthesize and liberate phage. Northrop (20) showed that *B. megatherium* strain 899a could be cultured for 200 generations in the absence of added Mg^{++} and liberate no phage. The addition of Mg^{++} resulted in the cells releasing phage at the end of an hour. He also found that the growth in various nutrient media played a part in determining whether the cells did or did not form phage. Clarke (75) noted that lysogenic *B. megatherium* when cultured in a synthetic medium, lost its lysogenicity after many transfers and proved sensitive to the parent strain's bacteriophage. This system in some ways is similar to the Kappa system in *Paramecium*. Under certain experimental conditions, *Paramecium* can be made to lose the "killer-substance," at which time the organisms become susceptible to its action.

Lwoff and co-workers (76, 77) showed that by treatment with ultraviolet light or by adding sulfhydryl compounds to the medium, practically all the cells of *B. megatherium*, strain 899, would form about 100 to 150 phage

particles per cell and lyse. The ultraviolet effect has been confirmed for *E. coli*, strain K12 (30), but does not appear to have any effect on *E. coli* strain "Li" (74). The effect of ultraviolet light (78) and sulfhydryl compounds (79) in inducing strain 899 to form phage has been found to depend upon the cationic balance of the bacteria.

Lwoff has suggested that in lysogenic bacteria the virus exists in the prophage stage (72). Owing to certain stimuli, the Co^{++} and Cu^{++} balance of the cell is upset, resulting in the replacement of Co^{++} by Cu^{++}. This development leads to the maturation of the prophage into an infective particle which is released by cellular lysis (79).

Latarjet (80), using ultraviolet irradiation methods, has estimated that there are three prophages in *E. coli* lysogenic strain K12. Jacobs (81) has found a similar number in lysogenic *Pseudomonas pyocyanea* strain P12/8A. Lwoff & Gutman have presented evidence that the prophage must multiply in the cell (72).

The nature of the prophage is of course of fundamental interest. This reviewer feels that in the light of the recent results of Hershey & Chase (12) the prophage may be a complex of phage specific DNA's. If this is so, then the prophage of lysogenic bacteria would be the same as the noninfective form found when phages attack susceptible cells such as *E. coli* B. If the prophage is a complex of phage specific DNA's and if lysogenicity in strain K12 is segregated as the bacterial genes are during crosses (82), then the phage specific DNA's must be looked upon as a gene-like structure. This hypothesis would certainly not be out of line with the genetic evidence which indicates that the phage behaves in many ways like a chromosome (58). The synthesis of the phage particle could then be looked upon as an example of the phage DNA's (genes) controlling enzymes whose activity is essential for the development of the intact virus.

Boyd (83, 84), working with a lysogenic strain of *Salmonella typhimurium*, however, has suggested a different mechanism leading to the formation of phage in lysogenic bacteria. He believes that phage formation is due to a mutation of the "symbiotic phage" to a lytic phage, rather than a maturation of an immature phage into an infective phage particle. Space does not permit going into his various reasons for this belief in the "mutation theory," and the reader is referred to the original papers for his views.

Biochemical analysis of lysogenic bacteria and those capable of becoming lysogenic, have revealed certain fundamental differences from infected *E. coli B* or *S. muscae* cells. When lysogenic *B. megatherium* or *P. pyocyanea* are induced to liberate phage or when susceptible *B. megatherium* or *P. pyocyanea* are infected the following events occur (85, 86). There is a synthesis of RNA, bacterial enzymes, and an increase in total cell material up to the time the cells lyse. Such infected cells can also form adaptive enzymes. As in *E. coli* B and *S. muscae*, however, there is a complete inhibition in DNA synthesis followed by a new rise in DNA synthesis which takes place at a faster rate than the increase in total cell material. A nuclear study of these types of

infected cells would be extremely interesting in view of Luria's hypothesis (62) referred to previously.

The Relationship between Bacterial Viruses and Animal Viruses

Although the synthesis of animal viruses may be different from that of bacterial viruses, recent work indicates that, at least in some respects, they are very similar. Work on the viruses of influenza, vaccinia, psitticosis-lymphogranuloma, and poliomyelitis have shown that they appear to lose their infectivity and take on new characteristics before beginning to multiply. This has best been shown for the influenza virus (87, 88). The data on influenza virus which indicates that multiplicity of reactivation (89) and recombination (90) may occur are very similar to those from the phage studies. It seems to this reviewer that with the tissue culture techniques it should be possible to study the adsorption process with animal viruses. Indeed, for many years the literature has been filled with observations that certain tissues will or will not support the growth of certain animal viruses. As far as this author is aware, there have been no studies to see whether this block takes place at the adsorption stage or some latter stage in virus synthesis. It would seem that such studies could be carried out and would be greatly desirable. The recent animal virus tissue culture assay technique of Dulbecco (91) should greatly facilitate such investigations.

Another similarity between animal and bacterial viruses may be found in the interference phenomenon. Thus, certain animal viruses will inhibit the multiplication of other animal viruses, just as certain phages will prevent the reproduction of other bacterial viruses. It is also possible that some of the so-called "masked" animal viruses are similar to the "incomplete" viruses found in certain lysogenic bacteria.

Summary

Using the reproduction of T2 in *E. coli* B, which in some ways is the simplest system to study, the formation of a phage particle may be summarized in the following manner.

The phage is adsorbed to the susceptible cell. This is a two-step reaction, the first being reversible, the second step leading to an irreversible attachment. The latter step results in some protein part of the phage, perhaps the tail, damaging the cell membrane. This permits the inner contents of the phage head consisting of DNA to pass into the cell. From the available data, it is also possible that a small part of the phage besides the DNA also enter the cell and plays a specific role in phage synthesis. Most of the phage membrane remains on the surface of the infected cell. The DNA isolated from the head of the phage is not adsorbed to the cell. One of the roles of the phage protein, therefore, is to get the DNA into the cell. These results explain why the phage cannot be detected by infectivity measurements after invasion of

the cell. On entering the cell, the DNA appears to form an organized intracellular structure, perhaps by combining with some bacterial component. Indeed the phage takes on many of the characteristics of the host. The bacterial nucleus is destroyed. The virus DNA assumes directional control of the energy system and enzymes of the host cell which were operating at the time the cell was infected. Under this control, the cell enzymes synthesize nonspecific components which at a later time are formed into virus specific materials under the influence of the virus DNA. The mechanism of this latter step is completely unknown.

Protein is synthesized from the time of infection. This is presumably the head membrane and the tail of the phage, since these two phage components comprise all the protein in the phage. After a lapse of about 5 to 7 min. there is an increase in purine-pyrimidine materials followed a few minutes later by an increase in DNA. The increase in DNA occurs arithemetically and precedes the formation of the infective particle by a few minutes. The increase in phage is also arithmetic rather than logarithmic.

Recent findings have translated these chemical events into morphological structures. The first phage specific material found before the infective particle can be detected are round collapsible heads which are the same size as the head of the infective particle and contain little if any DNA, but do have some phage antigenic specificity (92).[3] These results are of interest in view of the recent observations of Maaløe & Stent (94). Using P^{32} they found no measurable phosphorus containing materials with phage specificity besides the active phage particles. This is further support for the view that the tailless immature particles and other phage specific materials, besides the infective particle, probably contain little if any nucleic acid.

The above results indicate that phage reproduction takes place in steps. The various parts of the phage appear to be made separately, but not all components are synthesized at the same time. Before the intact phage particle is put together most of the various "pieces" necessary for the number of particles to be released by the cell are formed.[4] It may be that the time lapse between the DNA synthesis and formation of the infective particle is the time required to fix the tail onto the head. This last point is purely speculative, since it is not known exactly when the tail is made and no tails have been isolated at a time when there were no complete phage particles formed.

Luria's hypothesis of phage reproduction based on the phenomena of the

[3] Rountree (93) has also studied phage reproduction by immunological methods. Hershey's findings that the phage antigen remains on the cell surface on infection, together with Kozloff's observations which indicate that the phage protein is broken down, makes her experiments very difficult to interpret.

[4] For example, before any of the heads received their full amount of DNA, all the collapsible heads are formed that will be required for the number of phages liberated. This statement is based on the observation that the number of collapsible heads observed in cells, broken up before any infective phage is detected, is about that required for the number of "phages" liberated by control cells (92).

multiplicity of reactivation between ultraviolet inactivated particles (95), which was interpreted in terms of genetic damage and repair by recombination, no longer fits the new facts found by Dulbecco (96). There is no longer any evidence for the independent formation of genetic subunits and their reassembly into intact phage.

In conclusion, two points should be mentioned. Firstly, the above scheme does not hold for all different phages. For example, there is some evidence that the formation of T3 may be different from that of T2 (97). Secondly, the recent results on phage systems have brought forth more evidence for the similarity between phage formation and the transformation of bacteria by specific desoxyribonucleic acids.

LITERATURE CITED

1. Cohen, S. S., *Bact. Revs.*, **13,** 1 (1949)
2. Anderson, T. F., *Botan. Rev.*, **15,** 464 (1949)
3. Anderson, T. F., *Ann. Rev. Microbiol.*, **4,** 21 (1950)
4. Fong, J., *Ann. Rev. Microbiol.*, **3,** 423 (1949)
5. Benzer, S., Delbrück, M., Dulbecco, R., Hudson, W., Stent, G. S., Watson, J. D., Weidel, W., Weigle, J. J., and Wollman, E. L., *Viruses 1950* (California Institute of Technology, Pasadena, Calif., 100 pp., 1950)
6. Luria, S. E., *Science*, **111,** 507 (1950)
7. Putnam, F. W., *Science*, **111,** 481 (1950)
8. Puck, T. T., Garen, A., and Cline, J., *J. Exptl. Med.*, **93,** 65 (1951)
9. Garen, A., and Puck, T. T., *J. Exptl. Med.*, **94,** 177 (1951)
10. Herriott, R. M., *J. Bact.*, **61,** 752 (1951)
11. Herriott, R. M. (Personal communication)
12. Hershey, A. D., and Chase, M. J., *J. Gen. Physiol.* (In press)
13. Doermann, A. H., *Carnegie Inst. Wash. Yearbook*, **47,** 176 (1948)
14. Doermann, A. H., *J. Gen. Physiol.*, **35,** 645 (1952)
15. Anderson, T. F., and Doermann, A. H., *J. Gen. Physiol.*, **35,** 657 (1952)
16. Putnam, F. W., and Kozloff, L. M., *J. Biol. Chem.*, **182,** 243 (1950)
17. Kozloff, L. M., *J. Biol. Chem.*, **194,** 83 (1952)
18. Lesley, S. M., French, R. C., Graham, A. F., and van Rooyen, C. F., *Can. J. Research*, [E]**29,** 128 (1951)
19. French, R. C., Lesley, S. M., Graham, A. F., and van Rooyen, C. F., *Can. J. Research*, [E]**29,** 144 (1951)
20. Northrop, J. H., *J. Gen. Physiol.*, **34,** 471 (1951)
21. Anderson, T. F., *Trans. N. Y. Acad. Sc.*, **13,** 130 (1951)
22. Delbrück, M., and Luria, S. E., *Arch. Biochem.*, **1,** 111 (1942)
23. Delbrück, M., *J. Bact.*, **50,** 151 (1945)
24. Hershey, A. D., *Cold Spring Harbor Symposia Quant. Biol.*, **11,** 67 (1946)
25. Delbrück, M., and Bailey, W. T., *Cold Spring Harbor Symposia Quant. Biol.*, **11,** 33 (1946)
26. Adams, M. H., *J. Immunol.*, **66,** 131 (1951)
27. Adams, M. H., *J. Immunol.*, **67,** 313 (1951)
28. Dulbecco, R., *J. Bact.*, **63,** 209 (1952)
29. Herriott, R. M. (Personal communication)
30. Weigle, J. J., and Delbrück, M., *J. Bact.*, **62,** 301 (1951)

31. Novick, A., and Szilard, L., *Science*, **113**, 34 (1951)
32. Hershey, A. D., and Davidson, H., *Genetics*, **36**, 667 (1951)
33. Cohen, S. S., *Cold Spring Harbor Symposia Quant. Biol.*, **12**, 35 (1947)
34. Price, W. H. (Unpublished data)
35. Kozloff, L. M., Knowlton, K., Putnam, F. W., and Evans, E. A., *J. Biol. Chem.*, **188**, 101 (1951)
36. Evans, E. A., *Bact. Revs.*, **14**, 210 (1950)
37. Price, W. H., *J. Gen. Physiol.*, **34**, 231, 251 (1950)
38. Barry, G. T., and Goebel, W. F., *J. Exptl. Med.*, **94**, 387 (1951)
39. Raff, R. N., and Cohen, S. S., *J. Bact.*, **60**, 69 (1950)
40. Cohen, S. S., and Arbogast, R., *J. Exptl. Med.*, **91**, 607 (1950)
41. Herriott, R. M., *J. Gen. Physiol.* (In press)
42. Marshak, A., *Proc. Natl. Acad. Sci. U. S.*, **37**, 299 (1951)
43. Weed, L. L., and Cohen, S. S., *J. Biol. Chem.*, **192**, 693 (1951)
44. Wyatt, G. R., *Biochem. J.*, **47**, VII (1950)
45. Cohen, S. S., *Bact. Revs.*, **15**, 131 (1951)
46. Cohen, S. S., and Arbogast, R., *J. Exptl. Med.*, **91**, 637 (1950)
47. Price, W. H., *J. Gen. Physiol.*, **33**, 17 (1949)
48. Ajl, S. J., *J. Bact.*, **60**, 393 (1950)
49. Price, W. H., *Ann. Rept. to Rockefeller Inst.* (1950)
50. Pardee, A. B., *J. Gen. Physiol.*, **34**, 619 (1951)
51. Putnam, F. W., and Kozloff, L. M., *Science*, **108**, 386 (1948)
52. Evans, E. A., Putnam, F. W., and Miller, D., *Federation Proc.*, **10**, 181 (1951)
53. Labaw, L. W., *J. Bact.*, **62**, 169 (1951)
54. Labaw, L. W., *Proc. Natl. Acad. Sci. U. S.*, **37**, 721 (1951)
55. Koch, A. L., *Federation Proc.*, **10**, 210 (1951)
56. Evans, E. A., *Bact. Revs.*, **14**, 210 (1950)
57. Hershey, A. D., Kamen, M. D., Kennedy, J. W., and Gest, H., *J. Gen. Physiol.*, **34**, 305 (1951)
58. Hershey, A. D., and Rotman, R., *Genetics*, **34**, 47 (1949)
59. Maaløe, O., and Watson, J. D., *Proc. Natl. Acad. Sci. U. S.*, **37**, 507 (1951)
60. Kozloff, L. M., *J. Biol. Chem.*, **194**, 95 (1952)
61. Racker, E., *Nature*, **167**, 408 (1951)
62. Luria, S. E., *Federation Proc.*, **10**, 582 (1951)
63. Labaw, L. W., Mosley, V. M., and Wyckoff, R. W. G., *J. Bact.*, **60**, 511 (1950)
64. Herriott, R. M., *J. Gen. Physiol.*, **34**, 761 (1951)
65. Luria, S. E., and Human, M. L., *J. Bact.*, **59**, 551 (1950)
66. Price, W. H., *J. Gen. Physiol.*, **35**, 409 (1952)
67. Northrop, J. H., *J. Gen. Physiol.*, **35**, 471 (1952)
68. Heagy, F. C., *J. Bact.*, **59**, 364 (1950)
69. Watson, J. D., *J. Bact.*, **60**, 697 (1950)
70. Delbrück, M., *J. Bact.*, **50**, 131 (1945)
71. Murray, R. G. E., Gillen, D. H., and Heagy, F. C., *J. Bact.*, **59**, 603 (1950)
72. Lwoff, A., and Gutman, A., *Ann. inst. Pasteur*, **78**, 711 (1950)
73. Clarke, N. A., and Cowles, P. B., *J. Bact.*, **63**, 177 (1952)
74. Bertani, G., *J. Bact.*, **62**, 293 (1951)
75. Clarke, N. A., *J. Bact.*, **63**, 187 (1952)
76. Lwoff, A., Siminovitch, L., and Kjelgaard, N., *Ann. inst. Pasteur*, **79**, 815 (1950)
77. Lwoff, A., and Siminovitch, L., *Compt. rend.*, **232**, 1146 (1951)

78. Lwoff, A., and Huybers, K. (Unpublished data)
79. Lwoff, A., *Compt. rend.*, **234**, 366 (1952)
80. Latarjet, R., *J. Gen. Microbiol.* (In press)
81. Jacobs, F., *Ann. inst. Pasteur* (In press)
82. Lederberg, J. (Unpublished data)
83. Boyd, J. S. K., *J. Path. Bact.*, **62**, 501 (1950)
84. Boyd, J. S. K., *J. Path. Bact.*, **63**, 445 (1951)
85. Siminovitch, L., and Rapkine, S., *Compt. rend.*, **232**, 1605 (1951)
86. Jacobs, F., *Compt. rend.*, **232**, 1780 (1951)
87. Hoyle, L., *Brit. J. Exptl. Path.*, **29**, 390 (1948)
88. Henle, W., and Henle, G., *J. Exptl. Med.*, **90**, 23 (1949)
89. Henle, W., and Liu, O. L., *J. Exptl. Med.*, **94**, 305 (1951)
90. Burnet, F. M., and Lind, P. E., *J. Gen. Microbiol.*, **5**, 59 (1951)
91. Dulbecco, R., *Science*, **115**, 481 (1952)
92. DeMars, R. I., and Luria, S. E., *Bact. Proc.*, 70 (1952)
93. Rountree, P. M., *Brit. J. Exptl. Path.*, **32**, 341 (1951)
94. Maaløe, O., and Stent, G. S., *Acta Path. Microbiol. Scand.*, **30**, 149 (1952)
95. Luria, S. E., *Proc. Natl. Acad. Sci. U. S.*, **33**, 253 (1947)
96. Dulbecco, R., *J. Bact.*, **63**, 199 (1952)
97. Benzer, S., *J. Bact.*, **63**, 59 (1952)

THE STANDARDIZATION OF IMMUNOLOGICAL SUBSTANCES[1]

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Introduction

Toxins, toxoids, vaccines, and sera cannot, as yet, be identified chemically with sufficient precision to permit a statement, in gram units, of the fraction of pure substance contained in a preparation. Standardization tries to cope with this situation by introducing another unitage system, based on the specific biological activity of a given weight of an admittedly impure standard preparation.

The potency of some other preparation of the same substance is evaluated, in terms of units, by performing a biological assay, i.e. by comparing the specific responses produced by this unknown preparation and by the standard preparation in two similar biological test systems. Many papers dealing with biological standardization are concerned only, or mainly, with the statistical aspects of biological assays: the selection of suitable experimental designs, and of efficient methods for computing the estimates of potency and of confidence limits. Irwin (1) and Finney (2) have lately given short accounts of the development of statistical methodology in this field, particularly mentioning the contributions by Burn (3), Trevan (4), Gaddum (5), Bliss & Marks (6), Fieller (7, 8), and Wood (9, 10) to which must be added the important work by Irwin (11, 12) and Finney (2, 13) themselves. Perry (45), Armitage & Allen (46), and Miller (67) have recently contributed to the analysis of quantal response assays. Bibliographies were given in 1943 by Bliss & Cattell (14), and, covering the period from 1943 to 1947, by Armitage (15). The technique developed by the combined efforts of so many distinguished statisticians has reached a level of perfection which contrasts with the imperfection of the biological foundations of the immunological assays to which these techniques are applied.

We propose, in this survey, to present a discussion of this aspect of our subject, as in our opinion a revision of the basis of immunological standardization is now most needed. The fundamental problems become apparent when an attempt is made to define the terms potency and unit, and we may appropriately begin the general discussion with a seemingly innocent question: what do we imply when stating that immunological preparations have such and such potencies?

What is potency?—We may determine the amount of a toxic culture filtrate which, when administered in a prescribed way, will kill about 50 per cent of our test animals; this amount we may refer to as LD50 or the toxic

[1] The literature pertaining to this review was concluded in February, 1952.

potency of the preparation. Or we may determine the amount of a vaccine which, under given experimental conditions, will protect approximately 50 per cent of our test animals against a specified challenge dose of a given strain of bacteria; this amount may be called ED50, or the immunizing potency of the vaccine. In these and similar examples, we use the term potency for the quantity (in ml. or mg.) of a biological preparation which produces a certain effect. Potency, thus defined, is sometimes called absolute potency.

Experimental conditions, however, vary from time to time and from one laboratory to another; bacterial strains differ in virulence, animal strains differ in susceptibility, and even animals of the same strain respond differently in different climates or when fed different diets. It is clear therefore that statements of absolute potencies, though important in the daily work of individual laboratories, cannot have general validity. It was early recognised that the way out of the difficulties, arising from these inescapable variations in experimental conditions, would be the adoption of standard preparations, in comparison with which the potencies of other samples of the same substances could be determined. Defining the substances under consideration as "substances which exhibit specific biological properties, often unique and quite characteristic, which can be demonstrated by means of animals," Hartley (16), in a retrospective survey of international biological standardization, described the principle of potency measurement in comparison with a standard preparation, in the following way:

> Given two samples of one of these substances then their relative potency can be estimated by determining how much of each is required to produce the same biological effect. The one essential condition for the successful performance of these potency determinations is that the biological tests upon which they are based shall be carried out under strictly comparable conditions.

By this description, the relative potency of a biological preparation is defined as the ratio of the amounts of standard preparation and of test preparation which produce identical biological effects.

As standardization aims at making potency determinations carried out in different laboratories directly comparable, it seems obvious that we must sacrifice absolute potencies, which have no general validity, and instead should content ourselves with determinations of relative potencies. If everybody can be made to use the same standard preparation, then statements of relative potencies of test preparations in terms of this standard become universally current.

The standardization of an immunological substance thus seems quite simple. A suitable batch of a preparation containing the substance is labelled "standard preparation" and used as reference in comparative assays of test preparations, the relative potencies of which we wish to determine. The same principle applies, whether a local standard is adopted for research purposes, or an authoritative international standard is established by the Expert Committee on Biological Standardization of the World Health Organization.

This stage was reached for diphtheria antitoxin in 1922, when the first international unit was adopted by the Health Organization of the League of Nations. Other substances followed, and it would seem that only technical problems remained: the establishment of simple and reliable assay methods, and the development of the statistical superstructure of computation. However, both immunologists and statisticians were making fundamental reservations (1, 2, 3, 4, 6, 11, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 30, 31) concerning the validity of biological assays. These reservations had one main origin: the assumption (made when defining relative potency) that we are dealing with two samples of the same substance. Since, however, immunological substances cannot be identified chemically, we cannot make sure whether we are dealing with two samples of the same substance or not. This difficulty turns up whenever the fundamental conceptions of biological standardization are logically examined.

What is a unit?—Ehrlich (27, 28), who first developed this concept, defined a unit as the amount of active substance in a given amount of a standard preparation. Hartley (16) stated that "a unit is properly defined as the specific biologic activity contained in a given weight of the standard preparation." Miles (32) states that the League of Nations Health Organization, in 1922, defined the unit of potency as a certain weight of a dried standard preparation. The uncertainty as to whether a unit is to be defined as the amount of standard itself, or as the biological activity of this amount, was settled, in 1950, by Irwin (1) who explained:

> The unit is defined as the specific biological activity of a given amount of the standard. It cannot be defined as the given amount itself, because we may want to assay against the standard substances which exhibit the "specific activity" but are not necessarily in the same chemical form.

And in 1951, Miles (26) adds:

> The biological standard is almost completely analogous with physical standards, like that of length. The desired measurement—unit of potency in the one, and unit length in the other—is arbitrarily agreed upon as the property of a unique piece of material. The unit is the activity of a given weight or volume of a particular preparation of the biological substance in the one, and the distance between two marks on a metal bar in the other.

By these two explicit statements the fundamental flaw of the unit conception is exposed. The specific activity of the substances is not properly specified. Most of them exhibit several activities, each of which may be used for assaying. Thus toxic activity may mean both hemolytic activity for red cells and lethal activity for mice. Antitoxic activity may lead to toxin-antitoxin complexes which, on dilution, dissociate readily or practically not at all. Specific activity is not a single property shared by all substances bearing the same immunological name. It depends on the interaction between the substances and the biological test systems into which they are introduced. If the substances we want to assay are not identical, we may, by varying the

test systems, choose conditions of measurement which may favour the activity of either one or the other substance. Thus we may obtain different potency evaluations, in terms of units, when assaying the same preparations under different testing conditions.

The assumptions underlying a valid assay have been analyzed recently by Finney (2), Jerne & Wood (25), and Miles (26). The main point is that a valid determination of relative potency can be made only if, by suitable dilution in an inert diluent, we can make the standard preparation and the test preparations identical with respect to *all* biologically active constituents. It will be clear that this requirement is not likely to be met with unless the substances we want to compare have been prepared in very nearly the same way. Examples will show that the problem of choosing a standard is not only a difficult one, but one which often has no acceptable solution. Miles (32) furthermore points out that it is not sufficient, in order to make the assay valid, to ensure that the standard is homogeneous because heterogeneity of the material to be assayed will also effectively invalidate the assay.

From all points of view we are thus led to the same fundamental problems of standardization: the assumption of identity of standard and test preparations, and, in cases when this assumption must be rejected, the search for other ways of expressing potency. The importance of this point, which is often overlooked, has been brought forward with considerable force by Rasch (20). The problem is of a non-statistical nature and is particularly important for immunological standardization dealing, most often, with complicated, poorly defined mixtures of substances which may possess different active and inhibitive qualities, highly dependent on the source from which they were obtained and on the medium into which they are introduced.

The uncertainty, arising from this source, as to the validity of a determination of relative potency must be carefully kept in mind in every individual case. In comparison with this question, speculations as to the range of statistical confidence limits, based on assay variations, will often become altogether futile and unrealistic. Below we shall briefly discuss a number of actual cases. Though the issue is equally important for the standardization of other substances, such as alkaloids, vitamins, hormones and antibiotics, we shall limit ourselves to the immunological substances named in the first paragraph of this paper.

The three groups of substances, toxins, antitoxins, and toxoids, are closely interrelated. Antitoxins can only be demonstrated and measured by their actions on toxins, whereas the immunizing effect of toxoids can only be measured through the actions of the antitoxins produced. Measurements of antitoxins and toxoids thus involve antigen-antibody interaction and the production of antibody. Our lack of understanding of these fundamental immunological processes adds to the uncertainty to which the interpretation of such measurements must be subject.

Toxins

Among the immunological substances considered, only toxins have di-

rectly demonstable effects (lethal, dermonecrotic, hemolytic, etc.). It would, therefore, seem most simple to base the potency evaluation of toxins, toxoids, and antitoxins on standard preparations of highly purified toxins. Toxins, however, have never been internationally standardized, and it is interesting to note that the historical reason for this is precisely what we have called above the fundamental problem of standardization. International biological standardization actually started because of the revelation, by Ehrlich, of the complexity of diphtheria toxin. Ehrlich, in 1896, was confronted with the task of determining the potency of diphtheria antitoxic serum. The simple way first tried was to establish a unit of diphtheria toxin, the "minimum lethal dose," and to determine the amount of antitoxic serum which could neutralize a certain number of such lethal doses. Ehrlich showed that the reason for the failure of obtaining constant results by this procedure was the complexity of the toxin. Diphtheria toxin was shown to contain an unknown fraction of a substance (toxoid) which, though non-toxic, combined with antitoxin. Therefore, two samples of diphtheria toxin, which were alike with respect to toxicity, might be different with respect to combining power.

Although this is now a well known and almost trivial fact, we should like to emphasize the similarity between this situation and the fundamental problems arising in every case of immunological standardization. Let us imagine that we have a standard preparation of diphtheria toxin. We now want to determine the relative potency of a test preparation of diphtheria toxin differing from the standard in toxoid content. By performing a comparative assay of these two preparations we may find the ratio between the doses producing identical lethal or dermo-erythrogenic effect in guinea pigs or rabbits. However, by choosing another biological system of measurement, in this case the interaction with diphtheria antitoxin, we find another ratio between the concentrations of the two toxin preparations producing identical effects. Thus, by varying experimental conditions, we find different relative potencies.

Among bacterial toxins, the case of diphtheria toxin seems to be not only the first but also the simplest. After all, diphtheria toxin is thought to contain only one toxic factor which is the same for all preparations of this toxin. The only difficulty is the variable toxoid contents. Ehrlich thought this difficulty could be evaded by adopting a preparation of diphtheria antitoxin as standard preparation, in comparison with which the relative potency of other samples of diphtheria antitoxin could be determined. Reducing the serum selected to dry condition and preserving it *in vacuo* at low temperature, he called the amount of antitoxin contained in a fixed quantity (by weight) of this standard a unit. The present international unit for diphtheria antitoxin, which is 0.0628 mg. of the international standard preparation, was established on the same lines and should approximately equal the old Ehrlich unit. Ehrlich's solution of the problem of antitoxin standardization has been accepted as an achievement of great importance to science (16). It remains to be seen however, whether anything was actually gained by transferring the burden of standardization from toxin to antitoxin. The

implication that diphtheria antitoxin is a homogeneous, uniform, and stable substance, and that different sera containing this substance can be compared without trouble, is now known to be wrong to such an extent (24, 31) that it might have been more profitable to use a standard preparation of diphtheria toxin as a basis for measurement after all. It is now known that fresh diphtheria toxin can be stabilized by simple procedures before appreciable amounts of toxoid have been formed. If such a stable toxin were selected as standard, the situation would seem more simple, and we would be concerned mainly with differences and inhomogeneities among the antitoxins. In the section on sera this point will be further illustrated.

In principle, the same situation confronts us when assaying other toxins. For most bacterial toxins, however, the situation is much more complex on account of the variety of toxic factors present. Citing from the chapter on staphylococcus toxin in van Heyningen's monograph (33), we may gather the following points concerning this substance. Besides producing three or four hemolytic toxins, the staphylococci produce a number of other active agents, including leucocidin, enterotoxin, coagulase, fibrinolysin, hyaluronidase, lipase, and protease. Alpha toxin lyses the red cells of a wide range of species, the order of susceptibility being rabbit, ox, sheep, human, guinea pig. Besides being hemolytic, alpha toxin is also dermonecrotic and lethal. Beta toxin appears to lyse only sheep and ox blood, although it has been reported to be feebly lytic to the blood of humans, rabbits, and guinea pigs (34, 35, 36). The fact that it is not dermonecrotic but erythrogenic has been confirmed by several authors. It was found that although it was not lethal to mice, it would kill rabbits (36). Beta toxin was shown to inhibit alpha toxin (37). Gamma toxin (38) lysed the cells of the following animals in the order shown: horse, rat, ox, guinea pig, sheep, man, rabbit. It was found to be only slightly dermonecrotic and per unit of hemolytic activity it was less toxic than alpha toxin. Delta toxin (39) is believed to be distinct from the alpha, beta and gamma toxins. Staphylococcus rabbit leucocidin (40, 41) destroys rabbit polymorphonuclear leucocytes. It is probably identical with the alpha toxin. The staphylococci produce an additional leucocidin (42, 43) which destroys human as well as rabbit leucocytes. Human leucocidin is present only in filtrates of young cultures of certain strains which may not produce much alpha toxin. (44).

It will be immediately clear from this fragmentary description of the activities of staphylococcus toxin that a biological assay of two toxin preparations, containing mixtures in different proportions of some or all of the toxic factors mentioned, may be altogether futile. By varying the experimental conditions of the assay, different relative potencies are bound to turn up. If a dry standard preparation of staphylococcus toxin were established, and if a unit were adopted corresponding to a certain weight of this preparation, the definition of this unit as the "specific activity" of this amount of standard toxin would require a very careful specification of the particular activity alluded to. Even if this activity could be specified with sufficient precision to single out only one toxic substance from the mixtures, a comparative assay

based on the measurement of this activity, exhibited by different toxin preparations containing the responsible substance, would probably fail because the preparations would not contain this substance in an inert medium. Other substances, present in different relative amounts, might inhibit or enhance the activity to be measured and might do so to a different extent under different experimental conditions.

Streptococcus, pertussis, dysentery, vibrion septicus, histolyticus, oedematiens, perfringens, botulinus, tetanus, and other bacterial toxins present similar problems which can be satisfactorily approached only by research aiming at a chemical description of the factors responsible for the toxic activities and of the mechanisms involved. The case of tetanus toxin is relatively simple. This toxin has been isolated and crystallised (47, 48). However, crude tetanus toxin preparations must contain at least two toxic components, or other factors must be of considerable importance (70), as may be seen from experiments carried out by Llewellyn Smith (49). For one preparation of tetanus toxin she found that the lethal dose for rabbits was 350 times the lethal dose for guinea pigs. For another preparation of tetanus toxin, however, the lethal dose for rabbits was shown to be only three times the lethal dose for guinea pigs. If one of these two toxin preparations were called the standard, then, in the language of biological assay, the results obtained by Llewellyn Smith would mean that the other preparation, when choosing guinea pigs as the biological test system, showed a relative potency differing by a factor of about 100 from the relative potency obtained when using rabbits.

The potency evaluation of snake venoms is subject to similar complexities (59 to 63). The case of tuberculin is a special one. This toxin does not normally produce a skin response; only individuals sensitized by previous exposure to tuberculosis infection respond. Fundamentally the same difficulties, however, confront the standardization of tuberculins. Different preparations in use, Old Tuberculin and various Purified Protein Derivative tuberculins, have been produced by very different methods and do not contain identical proportions of the same immunologically active substances (64 to 67, 114 to 117). They cannot, therefore, be assayed against a common standard preparation. International standards have been established for Old Tuberculin (1931) and for mammalian P.P.D. (1951).

Sera

The establishment of an international standard preparation for diphtheria antitoxin in 1922 was followed by the international adoption of standard preparations for other sera. Table I gives the year of establishment and the present size of the units in milligrams of dry standard serum.

These standard preparations are maintained, on behalf of the World Health Organization, at the State Serum Institute, Copenhagen, Denmark, and samples are distributed periodically to the central control laboratories of practically all countries of the world. Some of them, when the stock was exhausted, were replaced by new batches of serum and new units were es-

tablished, equal in potency (as far as could be ascertained) to the old units. Thus, international standardization of sera has already been carried out for almost a generation, and, to judge from the world wide acceptance, with a considerable measure of practical usefulness and success.

The international unit of diphtheria antitoxin still equals the original Ehrlich unit of 1897. As diphtheria antitoxin was thought to be a single homogeneous substance, the validity of assaying other preparations of this

TABLE I

INTERNATIONAL STANDARDS FOR ANTITOXINS

Standard Preparation	Unit in mg.	Year of Adoption
diphtheria antitoxin	0.0628	1922
tetanus antitoxin	0.3094	1928
anti-dysentery serum (shiga)	0.0500	1928
gas gangrene antitoxin (perfringens)	0.1135	1931
gas gangrene antitoxin (vibrion septique)	0.0974	1934
gas gangrene antitoxin (oedematiens)	0.2681	1934
staphylococcus-α-antitoxin	0.2376	1934
anti-pneumococcus serum type 1	0.0886	1934
anti-pneumococcus serum type 2	0.0894	1934
gas gangrene antitoxin (histolyticus)	0.2000	1935
gas gangrene antitoxin (sordelli)	0.1334	1938
anti-A blood grouping serum	0.3465	1950
anti-B blood grouping serum	0.3520	1950

substance against the standard seemed evident (50, 51). Already from the very first, however, some dissenting views were put forward. In 1900, Roux (52), while investigating the curative effect of diphtheria antitoxin on laboratory animals which had been given an injection of diphtheria toxin, found that the therapeutic powers of different sera did not correspond to their relative antitoxic potencies in units. Others (53 to 58) investigated a property of antitoxic sera, which was called avidity by Kraus (53) and which was variously described as the "rate of combination," the "slope of neutralization curves," the "therapeutic efficiency per antitoxic unit," etc. These investigations led to the establishment of a number of methods for measuring avidity. The most well known are those proposed by Glenny *et al.* (58, 68, 69), the "serum ratio" (or *in vivo/in vitro* ratio) and the "dilution ratio." The serum ratio is the ratio between the relative potency of an antitoxic serum measured in terms of standard antitoxin in a comparative assay in guinea pigs, and the relative potency determined for the same preparation by the *in vitro* flocculation method. The dilution ratio is the ratio between the relative potencies of an antitoxin measured by the intracutaneous method on two different levels of initial toxin concentration. It will be seen from these definitions that we are, in principle, back at the same fundamen-

tal difficulty as described for toxins; by varying the experimental conditions we find different relative potencies.

Jerne (31) analyzed the problem of diphtheria antitoxin standardization and found that the antitoxin first produced by an immunized animal differs from the international standard antitoxin to such an extent that, by varying the assay conditions, we may find relative potencies varying within a twenty fold range. Table II shows two measurements of diphtheria antitoxic serum by the intracutaneous rabbit method, showing a large discrepancy between the results arrived at when carrying out the assays on two different levels of initial concentration of the same toxin (71).

TABLE II

DISCREPANCY ENCOUNTERED WHEN DETERMINING THE RELATIVE POTENCY OF A SERUM OF LOW AVIDITY AT DIFFERENT TOXIN CONCENTRATIONS

Serum Concentration	Initial Toxin Concentration 32 Lf/l.		Initial Toxin Concentration 1 Lf/l.	
	Standard Serum	Horse Serum 2508	Standard Serum	Horse Serum 2508
4000	0*	—	0	—
3200	8	—	0	—
2500	16	—	0	—
2000	18	—	0	—
1600	21	—	0	—
1250	23	—	0	—
1000	—	—	0	—
800	—	0	0	0
630	—	0	0	0
500	—	0	0	0
400	—	0	0	0
320	—	5	0	0
250	—	9	0	0
200	—	12	0	0
160	—	14	0	5
125	—	16	5	8
100	—	16	9	10
80	—	17	12	12
63	—	18	13	13
50	—	19	14	14
40	—	20	15	14
32	—	22	15	15

* Mean diameter (in millimeters) of six rabbit skin responses to injections of 0.1 ml. mixtures of serum and toxin.

The serum concentrations given are milliliters horse serum 2508 per liter of the mixtures, and international units of standard serum per 100 l. of the mixtures. The mixtures were incubated 2 hr. at 37°C. before injecting 0.1 ml. samples into rabbit skin.

The only way out of these difficulties is a closer investigation into the nature and properties of the substances with which we are dealing. Our experimental investigations into the diphtheria antitoxic properties of different sera led to the conclusion that diphtheria antitoxin is not a uniform substance, but a group of substances showing different degrees of affinity for diphtheria toxin. By studying the curves of neutralization of toxin by antitoxin in low concentrations, it was found that the behaviours of different antitoxic sera could be described by means of the association constants governing the basic reaction $T+A \rightleftarrows TA$. It should be emphasized that this description of behaviour is not an auxiliary device giving additional information about a serum besides the relative potency in international units, but that it has been shown that a general evaluation of diphtheria antitoxic sera in international units is intrinsically invalid and must be replaced by more satisfactory ways of expressing the neutralizing power of a serum. Jerne (31) suggests introducing two values: the molecular concentration of antitoxin in a serum, and the association constant describing the avidity of this particular sample of antitoxin towards toxin. This constant (K_1) has tentatively been named the "avidity constant." Potency, by this suggestion, is no longer a single valued constant but becomes the totality of all parameters necessary to describe the behaviour of a serum within the range of experimental conditions considered. This is a very ambitious plan which has no chance, at present, of gaining practical significance except for diphtheria antitoxin. The exceptional position of diphtheria antitoxin is due partly to the supposed homogeneity of the toxin but mainly to the knowledge accumulated by research into diphtheria toxin-antitoxin interaction based on *in vitro* experiments.

Eaton (72) and Pappenheimer (73) have reported the preparation of almost pure diphtheria toxin. The molecular weight was found to be about 70,000, the nitrogen contents about 16 per cent. Pappenheimer's flocculation experiments showed that the composition of the flocculi in the quickest flocculating mixture of a series was about two molecules of antitoxin to one molecule of toxin (TA_2). The amount of toxin flocculating in this manner per antitoxin unit is called one Lf. One Lf was found to contain approximately 0.00045 mg. N, and, if not toxoided, about 40 or 50 LD_{50} for guinea pigs weighing 300 gm. These data made it possible to suggest a combining mechanism, by replacing rather vague biological activities with physico-chemical concepts. Pappenheimer & Robinson (74) and Pappenheimer (75) showed that concentration of both antitoxin and toxin may be calculated from a single nitrogen determination on the precipitate at flocculation point, even though the strength of neither toxin nor antitoxin is known and no standard available. They found that precipitation at this point was complete, that no appreciable non-specific nitrogen is carried down with the precipitate, and that the ratio between antitoxin and toxin nitrogen was 3.5:1. Jerne (31) showed that the concentration and avidity constant of antitoxin in weaker sera that cannot be made to flocculate, could be estimated from a set of neutralization curves based on measurements, in rabbit skin,

of the concentration of free toxin in toxin-antitoxin mixtures. The interpretations of the experimental results given by these authors may still be inadequate. There can be no doubt, however, that diphtheria antitoxin is a group of substances exhibiting different antitoxic properties, and that a unitage based on a fixed standard preparation of diphtheria antixoic serum cannot represent a generally valid evaluation of relative potency.

If we turn our attention to antitoxins other than diphtheria, the situation is, again, more complicated. Toxins, containing variable amounts of different antigenic factors, give rise to equally complex antitoxins, and if we consider that this complexity is furthermore complicated by avidity, it becomes clear that statements of the relative potencies of such sera in international units should be treated with scepticism and that estimates of the statistical error appertaining to the assays on which such statements are based can be only of secondary importance.

The fundamental importance for standardization of the complexities described can always be demonstrated by showing the discrepancies between relative potencies measured under adequately different sets of experimental conditions.

The literature on serum antibody titration is too extensive to be comprehensively reviewed. Contributions to the avidity problem in connection with the potency evaluation of lamb dysentery and tetanus antitoxins were made by Glenny, Barr, Ross & Stevens (24), Ipsen (76), Llewellyn Smith (49) and Petrie (77). Most work on international standardization of various sera has been published in the *Bulletin of the Health Organization of the League of Nations*. The 1945 issue of this periodical contains a full bibliography of the contributions prior to that year (78). Among more recent work we may mention Hazra, Lahiri & Sokhey (62), Grasset (63), and Schöttler (118) on anti-snake-venoms, Heidelberger & DiLapi (80) on the measurement of antibodies in human sera, Barr (58) and Barr & Glenny (27) on avidity problems, Ramon & Richou (79) on diphtheria, tetanus, and staphylococcus titrations, Branham (81) on the standardization of dysentery antitoxin (shiga) and Miles (82) on the standardization of anti-A and anti-B blood grouping sera.

Toxoids and Vaccines

When going from the subject of serum potency determination to the discussion of the evaluation of the immunizing potency of toxoids and vaccines, we proceed towards increasingly complex situations. Mercier & Pillet (97) and Thiery & Richou (113) have recently considered the problems of staphylococcus toxoid standardization.

A considerable amount of effort has been spent on the problem of measuring the immunizing potency of diphtheria and tetanus toxoid. The first major contribution was that of Prigge (83, 84). More recently these problems have been discussed by Hartley (88), Greenberg, Gibbard & Morrell (85), Greenberg & Roblin (86), Cavalli (89), Levine, Wyman & Edsall (94), Jerne & Maaløe (87, 92), Carlinfanti (90), Holt (91), Lahiri (93), and Kurokawa *et al.* (95). Doubtless this question has practical importance, as these

toxoids are widely used and have proved effectively prophylactic against the diphtheria and tetanus diseases. The toxoids issued must be of high quality, and the laboratories in charge of toxoid control must therefore rely on a method of evaluation which may discriminate between good and bad products. On the other hand, the practical importance must not be overestimated, as a full course of immunization with any toxoid preparation, produced by a capable laboratory, can hardly escape affording ample protection. The question of toxoid standardization has been taken up by the Expert Committee on Biological Standardization of the World Health Organization and, in 1951, international standard preparations for tetanus toxoid and diphtheria toxoid, plain, were established (96).

The term "immunizing potency" seems to imply that we are not interested in the composition of the toxoid preparation, but only in its immunizing effect. The toxoid concentration in a preparation for human prophylaxis is always so large that an easily performed flocculation experiment can tell us the approximate toxoid contents in Lf. This, however, is not felt to yield sufficient information, since the immunizing effect depends not only on the toxoid itself but also on other constituents of the preparations which act as adjuvants or carrier substances. Thus, adsorption onto aluminium hydroxide or aluminium phosphate greatly enhances the immunizing power of a given dose of toxoid. As this effect depends not only on the chemical composition of the carrier material but also on physical properties (the way the aluminium hydroxide gel has been prepared), even an exact chemical analysis of a toxoid preparation, if this were posssible, would not enable us to predict the "immunizing potency." We must therefore carry out animal experiments, but a little consideration will show that such experiments are unlikely to give us satisfactory information. The standard toxoid preparation, used for reference in order to determine relative potency, may be very different from our test preparations in composition. We, therefore, do not know whether the immunizing properties of the preparations in our assays are directly comparable. Must we measure the degree of immunity attained four weeks after applying the immunizing stimulus, or shall we wait say twelve weeks before testing the resistance of the animals? One toxoid preparation may produce a quick response, while a different preparation may produce a slower response [Hartley (88)]. Perhaps we should wait still longer to control the lasting level of immunity [Holt (91)]. What do we understand by "immunizing effect" or "degree of immunity attained"? Is this the concentration of antibody in the circulating blood, or the degree of resistance of the animals against a toxic challenge, or perhaps, the ability to respond quickly and abundantly to a second antigen stimulus, or to an infection? These properties may vary independently so that the measurement of one of them does not necessarily supply information as to the others. If we want to measure the antitoxin in the circulating blood, we are confronted with all the difficulties of antitoxic potency evaluation which we have already described.

What dosage design shall we use? If our test animals are guinea pigs, we

must use only a fraction of a human dose. This means that we must either inject a fraction of a ml., as such, or we must inject a dilution of the preparation we are testing. Both procedures will cause a situation at the injection site which is perhaps significantly different from the situation in the human subcutis after injection of a many fold dose. Should we apply one injection or two injections, separated by a certain time interval, before testing the animals? The application of only one stimulus will bring out more marked differences between different preparations than the use of two injections, which, on the other hand, comes closer to the procedure followed in human prophylaxis. But can legitimate conclusions as to the prophylactic effect in humans be drawn from the measurement of guinea pig responses? It will be seen that all these difficult and, to a large extent, unanswerable questions will make the validity of any statements of relative immunizing potencies of toxoids in terms of units extremely doubtful. Still, comparative assays are being performed, under the assumption that resistance to a toxin challenge attained by immunized animals after a given time is a useful reflection of the immunizing power of the toxoid used. Table III shows the result of such an assay.

TABLE III

ATTEMPT TO ASSAY THE IMMUNIZING POTENCY OF AN ALUMINIUM HYDROXIDE ADSORBED TOXOID*

International Standard for Diphtheria Toxoid, Plain mg.	Guinea Pig Survival Quota to 20 LD_{50} of Diphtheria Toxin	Survival Percentage
0.05	0/28	0
0.16	3/28	11
0.50	14/29	48
1.60	11/27	41
5.0	16/25	64
16.0	28/30	93
0.005†	0/24	0
0.016†	0/29	0
0.05†	18/30	60
0.16†	22/23	96
0.50†	29/29	100
1.60†	24/24	100

* All injections were given in a volume of 1 ml. The toxin challenge was applied four weeks later.

† Milligrams toxoid absorbed on $Al(OH)_3$.

It will be seen that 5 mg. of the plain standard toxoid protected practically the same fraction of guinea pigs as did 0.05 mg. of the adsorbed toxoid. However, 1.6 mg. of the plain standard toxoid produced a much better effect than 0.016 mg. of the absorbed toxoid. This is a very frequent phenomenon in biological standardization, usually described as the lack of parallelism

between log dose/response curves. We shall not enter into the biomathematical language, but only emphasize the obvious fact that such results do not permit the evaluation of a relative potency. To decide, on the basis of the above assay, whether 0.5 mg. adsorbed toxoid were to be preferred to 10 mg. plain standard toxoid as a first dose human prophylactic would not only be difficult but impossible. The object of the assay, which was the evaluation of a relative immunizing potency, has not been attained. Nonparallelism is not always so conspicuous in this kind of assay as in the above example. Prigge (84) based the standardization of diphtheria toxid in Germany on this method of comparing different toxoids with a standard toxoid preparation. A certain amount, by weight, of this standard he named a protection unit (*Schutzeinheit*). Greenberg *et al.* (85, 86) also carry out the control of diphtheria and of tetanus toxoids by this method of assay in comparison with standard preparations. The Expert Committee of the World Health Organization has now decided to establish two international standard preparations for diphtheria toxoid, one plain and purified, and one adsorbed onto aluminium hydroxide (96). A satisfactory solution can be expected only if further research leads to increased knowledge of the process of immunization.

Most of what has been said of the standardization of toxoids applies to vaccines as well. We cannot reasonably hope to obtain a generally valid evaluation of the relative potency of vaccines in terms of protective units of a standard vaccine. Comparative assays in animals can give us data which are strictly valid only for the experimental conditions chosen. If, from such experimental results, we wish to draw conclusions as to the relative efficiency of vaccine preparations in human prophylaxis, such conclusions must partly depend on personal judgement derived from other experience and belief, which cannot be given an objective quantitative expression. The Expert Committee on Biological Standardization of the World Health Organization is at present considering the standardization of cholera and pertussis vaccines. In 1950, Sokhey & Habbu (98) published their method of assaying cholera vaccine, discussing previous attempts by Ranta & Dolman (99) and Burrows, Mather, Elliott & Havens (100). Sokhey & Maurice (101) have given a method of assaying plague vaccine. A great deal of work on the potency evaluation of pertussis vaccine has been done by Kendrick *et al.* (102), Pittman & Lieberman (103) and, lately, by Cockburn, Evans & Knowelden (104) and Krag Andersen (105). Ørskov (106) has discussed the possibility of standardizing smallpox vaccine. Powell & Jamieson (107) have tested the immunizing power of dysentery antigen in mice. The potency determination of typhoid vaccines has been attempted by Griffitts (108) and Luippold (109), and has recently been thoroughly investigated by Batson *et al.* (110) and Felix (111) who came to the conclusion that the assay of typhoid (and paratyphoid) vaccines against a standard vaccine has now become possible. Batson (112) discussed the hypotheses underlying the two current methods of assaying an immunizing agent. Either a fixed dose of a vaccine is given to all groups of animals, followed, after a certain time, by a graded series of challenge doses in order to find the size of challenge against

which 50 per cent of the animals are protected, or, by giving a graded series of vaccine doses to several groups of animals, the vaccine dose which will protect 50 per cent of the animals against a fixed challenge is determined. The assumptions underlying these procedures are that the size of the challenge against which the animals are protected, or the quantity of the vaccine which protects against a certain challenge, are directly related to the potency of the product. These hypotheses, however, can only be tested if the term "potency" is defined. If by potency we mean, for instance, the quantity of vaccine which protects 50 per cent of the animals, then we are dealing not with standardization, but with absolute potencies. If by potency we mean relative potency, we have to look for the ratio between the quantities of two preparations producing identical effects. This ratio should be independent of experimental conditions, otherwise, as we have seen, no generally valid relative potency exists. We are back at the fundamental requirement for valid determinations of relative potencies which has been the main theme of this review and the most important problem of immunological standardization.

Conclusions

The picture drawn above of the the present position of immunological standardization may seem too discouraging. International standardization of many sera and standardization, on a national scale, of some toxoids may be said to have been carried out successfully for many years already. There is no other measure of this success, however, than the satisfaction of the control laboratories. Antitoxic serum therapy has always been a doubtful business and has not, so far, enabled us to test the relative therapeutic efficiencies of different serum preparations. Toxoids, on the other hand, have proved so efficient that the presence of a reasonable quantity of the specific antigen, which may be tested by flocculation, is probably a sufficient criterion for a good prophylactic.

Standardization, that is the evaluation of relative potencies in units, however, depends by definition on the assumption of identity in composition of standard and test preparation. The situation is well described by Miles (26). "It is one of the contradictions in biological standardization that at the time when standards are of the greatest use, that is, when our chemical and physical knowledge of the active principle is inadequate, the assumptions are least likely to be true."

We fully realize, of course, that the basic difficulties of immunological standardization, which we have outlined in this review, are appreciated by many research workers. But among many other workers there is a tendency to regard standards, and especially international standards, as the authorized way out of the difficulties of potency evaluation. Standardization, however, is subject to all the limitations of our present lack of knowledge in immunology, and it can not, by itself, solve any fundamental problem in this field. Bearing these limitations in mind, the established standards can serve their rightful purpose of being internationally available reference preparations of immunological substances.

We may sum up this discussion by considering two basic situations. In the first situation we carry out assays of a test preparation in comparison with a standard under the assumption that the two preparations contain the same active substances in the same proportions. The simplifying assumption is made because of our lack of knowledge, and we should keep testing this assumption by measuring responses over a large range of varying experimental conditions and in different biological test systems.

If the basic assumption proves to have been too simple, we are in the second situation. Standard and test preparations are not directly comparable and a relative potency does not, therefore, exist. We must make other assumptions which, although more complicated, are more nearly related to the truth. This means that we must give up relative potency and look for a description, by means of a number of adequate constants, of the properties of the substances investigated.

LITERATURE CITED

1. Irwin, J. O., *J. Hyg.*, **48**, 215 (1950)
2. Finney, D. J., *J. Roy. Statistical Soc.*, Suppl. 9, 46 (1947)
3. Burn, J. H., *Physiol. Rev.*, **10**, 146 (1930)
4. Trevan, J. W., *Proc. Roy. Soc.* (*London*), [B] **101**, 483 (1927)
5. Gaddum, J. H., *Spec. Rept. Ser. Med. Research Council* (*London*), No. 183 (1933)
6. Bliss, C. I., and Marks, H. P., *Quart. J. Pharmacol.*, **12**, 82 (1939)
7. Fieller, E. C., *J. Roy. Statistical Soc.*, Suppl. **7**, 1 (1940)
8. Fieller, E. C., *Quart. J. Pharmacol.*, **17**, 117 (1944)
9. Wood, E. C., *Nature*, **153**, 84, 680 (1944)
10. Wood, E. C., *Nature*, **158**, 835 (1946)
11. Irwin, J. O., *J. Roy. Statistical Soc.*, Suppl. **4**, 1 (1937)
12. Irwin, J. O., *J. Hyg.*, **43**, 121 (1943)
13. Finney, D. J., *Probit Analysis* (Cambridge Univ. Press, London, England, 269 pp., 1947)
14. Bliss, C. I., and Cattell, M., *Ann. Rev. Physiol.*, **5**, 479 (1943)
15. Armitage, P., *J. Roy. Statistical Soc.*, **114**, 530 (1951)
16. Hartley, P., *Proc. Roy. Soc. Med.*, **39**, 45 (1945)
17. Bliss, C. I., *J. Am. Pharm. Assoc. Sci. Ed.*, **29**, 465 (1940)
18. Fieller, E. C., *Analyst*, **72**, 37 (1947)
19. Gaddum, J. H., *Biochem. J.*, **25**, 1113 (1931)
20. Rasch, G., *Biometrics Bull.*, **3**, 173 (1947)
21. Wood, E. C., *Analyst*, **71**, 1 (1946)
22. Ipsen, J., *Contribution of the Theory of Biological Standardization* (Nyt Nordisk Forlag, Copenhagen, Denmark, 248 pp., 1941)
23. Ipsen, J., *Z. Immunitätsforsch.*, **102**, 369 (1942)
24. Glenny, A. T., Barr, M., Ross, H. E., and Stevens, M. F., *J. Path. Bact.*, **35**, 495 (1932)
25. Jerne, N. K., and Wood, E. C., *Biometrics Bull.*, **5**, 273 (1949)
26. Miles, A. A., *Brit. Med. Bull.*, **7**, 283 (1951)
27. Barr, M., and Glenny, A. T., *J. Hyg.*, **47**, 107 (1949)
28. Ehrlich, P., *Klin. Jahrb.*, **6**, 299 (1896)
29. Dale, H. H., *Analyst*, **64**, 554 (1939)
30. Miles, A. A., *Analyst*, **73**, 530 (1948)
31. Jerne, N. K., *Acta. Path. Microbiol. Scand.*, Suppl. **87**, 183 pp. (1951)
32. Miles, A. A., *Bull. World Health Org.*, **2**, 205 (1949)

33. Heyningen, W. E. van, *Bacterial Toxins* (Blackwell Scientific Publication, Oxford, England, 133 pp., 1950)
34. Glenny, A. T., and Stevens, M. F., *J. Path. Bact.*, **40**, 201 (1935)
35. Bryce, L. M., and Rountree, P. M., *J. Path. Bact.*, **43**, 173 (1936)
36. Llewellyn Smith, M., and Price, S. A., *J. Path. Bact.*, **47**, 361 (1938)
37. Christie, R., and Graydon, J. J., *Australian J. Exptl. Biol. Med. Sci.*, **19**, 9 (1941)
38. Llewellyn Smith, M., and Price, S. A., *J. Path. Bact.*, **47**, 379 (1938)
39. Williams, R. E. O., and Harper, G. J., *J. Path. Bact.*, **59**, 69 (1947)
40. Neisser, M., and Wechsberg, F., *Z. Hyg. Infektionskrankh.*, **36**, 299 (1901)
41. Wright, J., *Lancet*, **I**, 1002 (1936)
42. Panton, P. N., and Valentine, F. C. O., *Lancet*, **II**, 506 (1932)
43. Valentine, F. C. O., *Lancet*, **I**, 526 (1936)
44. Proom, H. J., *J. Path. Bact.*, **44**, 425 (1937)
45. Perry, W. L. M., *Spec. Rept. Series Med. Research Council*, No. 270 (1950)
46. Armitage, P., and Allen, I., *J. Hyg.*, **38**, 298 (1950)
47. Pillemer, L., Wittler, R. G., Burrell, J. I., and Grossberg, D. B., *J. Exptl. Med.*, **88**, 205 (1948)
48. Pillemer, L., Grossberg, D. B., and Wittler, R. G., *J. Immunol.*, **54**, 213 (1946)
49. Llewellyn Smith, M., *Bull. Health Org., League of Nations*, **10**, 104 (1942, 1943)
50. Glenny, A. T., and Allen, K., *J. Path. Bact.*, **24**, 61 (1921)
51. Jensen, C., *Acta Path. Microbiol. Scand.*, Suppl. 14, 211 pp. (1933)
52. Roux, E., *Compt. rend. 10th congr. intern. hyg. et dém., Paris*, **21**, 156 (1900)
53. Kraus, R., *Zentr. Bakt. Parasitenk., I Orig.*, **34**, 488 (1905)
54. Morgenroth, J., *Z. Hyg. Infektionskrankh.*, **48**, 177 (1904)
55. Madsen, T., and Schmidt, S., *Z. Immunitätsforsch.*, **65**, 357 (1930)
56. Llewellyn Smith, M., *Bull. Health Org., League of Nations*, **7**, 739 (1938)
57. Glenny, A. T., Pope, C. G., and Waddington, H., *J. Path. Bact.*, **28**, 279 (1925)
58. Barr, M., *J. Path. Bact.* **56**, 85 (1949)
59. Kellaway, C. H., *Ann. Rev. Biochem.*, **8**, 541 (1939)
60. Grasset, E., Zoutendyk, A., and Schaafama, A., *Trans. Roy. Soc. Trop. Med. Hyg.*, **28**, 601 (1935)
61. Grasset, E., *Bull. Health Org., League of Nations*, **5**, 367 (1936)
62. Hazra, A. K., Lahiri, D. C., and Sokhey, S. S., *Bull. Health Org., League of Nations*, **12**, 384 (1945, 1946)
63. Grasset, E., *Bull. World Health Org.*, **2**, 69 (1949)
64. Seibert, F. B., *Chem. Revs.*, **34**, 107 (1944)
65. Long, D. A., Miles, A. A., and Perry, W. L. M., *World Health Org. Memorandum, BS/120* (1951)
66. Green, H. H., *World Health Org. Memorandum, BS/126* (1951)
67. Miller, L. C., *Ann. N. Y. Acad. Sci.*, **52**, 903 (1950)
68. Glenny, A. T., and Llewellyn-Jones, M., *J. Path. Bact.*, **34**, 110 (1931)
69. Glenny, A. T., and Barr, M., *J. Path. Bact.*, **35**, 91 (1932)
70. Miles, A. A., *Bull. World Health Org.*, **2**, 59 (1949)
71. Jerne, N. K., *World Health Org. Memorandum, BS/77* (1950)
72. Eaton, M. D., *J. Bact.*, **31**, 347 (1936)
73. Pappenheimer, A. M., Jr., *J. Biol. Chem.*, **120**, 543 (1937)
74. Pappenheimer A. M., Jr., and Robinson, E. S., *J. Immunol.*, **32**, 291 (1937)
75. Pappenheimer, A. M., Jr., *J. Bact.* **43**, 273 (1942)
76. Ipsen, J., *Z. Immunitätsforsch.*, **102**, 347 (1942)
77. Petrie, G. F., *Bull. Health Org., League of Nations*, **10**, 113 (1942, 1943)

78. *Bull. Health Org., League of Nations*, **11**, 1 (1945)
79. Ramon, G., and Richou, R., *Rev. immunol.*, **14**, 161 (1950)
80. Heidelberger, M., and DiLapi, M. M., *J. Immunol.*, **61**, 153 (1949)
81. Branham, S. E., *Bull. World Health Org.*, **4**, 111 (1951)
82. Miles, A. A., *Bull. World Health Org.*, **3**, 301 (1950)
83. Prigge, R., *Arb. Staatsinst. exptl. Therap. u. Georg Speyer-Hause Frankfurt a. M.*, **32**, 1 (1935)
84. Prigge, R., *Ergeb. Hyg. Bakt. Immunitätsforsch. Exptl. Therap.*, **22**, 1 (1939)
85. Greenberg, L., Gibbard, J., and Morrell, C. A., *Bull. Health Org., League of Nations*, **12**, 365 (1945, 1946)
86. Greenberg, L., and Roblin, M., *J. Immunol.*, **59**, 221 (1948)
87. Jerne, N. K., and Maaløe, O., *Bull. World Health Org.*, **2**, 49 (1949)
88. Hartley, P., *Proc. Roy. Soc. Med.*, **38**, 473 (1945)
89. Cavalli, L. L., *Boll. ist. sieroterap. milan.*, **26**, 216 (1947)
90. Carlinfanti, E., *J. Immunol.*, **64**, 373 (1950)
91. Holt, L. B., *Developments in Diphtheria Prophylaxis* (William Heinemann, London, England), 181 pp. (1950)
92. Jerne, N. K., *World Health Org. Memoranda, BS/68, 79, 113* (1949, 1950, 1951)
93. Lahiri, D. C., *Indian J. Med. Research*, **39**, 229 (1951)
94. Levine, L., Wyman, L., and Edsall, G., *J. Immunol.*, **63**, 219 (1949)
95. Kurokawa, M., Murata, R., Nakano, T., Yadada, T., and Kubota, K., *Japan. Med. J.*, **4**, 55 (1951)
96. Expert Comm. on Biol. Standardization, *World Health Org. Rept. 5th Session* (In press)
97. Mercier, P., and Pillet, J., *Bull. World Health Org.*, **2**, 45 (1949)
98. Sokhey, S. S., and Habbu, M. K., *Bull. World Health Org.*, **3**, 43 (1950)
99. Ranta, L. E., and Dolman, C. E., *Can. J. Pub. Health*, **35**, 473 (1944)
100. Burrows, W., Mather, H. N., Elliott, M. E., and Havens, I., *J. Infectious Diseases*, **81**, 157 (1947)
101. Sokhey, S. S., and Maurice, H., *Bull. office intern. hyg. publ.*, **27**, 1534 (1935)
102. Kendrick, P. L., Updyke, E. L., and Eldering, G., *Am. J. Pub. Health*, **39**, 179 (1949)
103. Pittman, M., and Lieberman, J. E., *Am. J. Pub. Health*, **38**, 15 (1948)
104. Cockburn, W. C., Evans, D. G., and Knowelden, J., *Brit. Med. J.*, **I**, 1464 (1951)
105. Krag Andersen, E., *World Health Org., Memorandum, BS/123* (1951)
106. Ørskov, J., *Bull. Health Org., League of Nations*, **7**, 679 (1938)
107. Powell, H. M., and Jamieson, W. A., *J. Bact.*, **54**, 371 (1947)
108. Griffitts, J. J., *U. S. Pub. Health Service, Pub. Health Repts.*, **59**, 1515 (1944)
109. Luippold, G. F., *Am. J. Pub. Health*, **35**, 153 (1945)
110. Batson, H. C., Brown, M., and Oberstein, M., *U. S. Public Health Service, Pub. Health Repts.*, **66**, 789 (1951)
111. Felix, A., *J. Hyg.*, **49**, 268 (1951)
112. Batson, H. D., *J. Immunol.*, **66**, 737 (1951)
113. Thiery, J. P., and Richou, R., *Bull. off. intern. épizooties*, **33**, 304 (1949)
114. Jensen, K. A., Bindslev, G., Möller, S., Hansen, A., and Lind, P., *Tubercle*, **19**, 386, 433 (1938)
115. Doig, A. T., Gemmill, G., Gregory Kayne, G., Linggood, F. V., Parish, H. J., and Westwater, J. S., *Brit. Med. J.*, **I**, 992 (1938)
116. Seibert, F. B., and Glenn, J. T., *Am. Rev. Tuberc.*, **44**, 9 (1941)
117. Holm, J., and Lind, P., *U. S. Pub. Health Service, Pub. Health Repts.*, **62**, 6, 188 (1947) (Extracts)
118. Schöttler, W. H. A., *Bull. World Health Org.*, **5**, 293 (1952)

ASSOCIATIONS BETWEEN MICROORGANISMS AND HIGHER PLANTS (MYCORRHIZA)[1]

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The work done in recent years upon the association between roots of higher plants and microorganisms has proved to be of fundamental importance in various branches of botanical study: in the study of plant pathology, of soil activity, and of mycorrhiza. In particular there has been a tendency to emphasise the differences between those populations of microorganisms to be found in the root regions of plants and those in the soil. There are numerous reviews already published, such as that of Katznelson and his colleagues (1), covering the subject in general. The emphasis on living root surfaces is understandable since these are of interest to all those engaged in the study of the growth and health of plants in natural conditions or in cultivation. Nevertheless the root regions of living plants are by no means the only series of sites bearing populations strikingly different from the remainder of the soil either in types or numbers of organisms. This point has been made by Smith (2), Harley (3), and Garrett (4) who have approached the subject from different angles, and its importance will become more apparent in the course of this article.

Garrett (5) has stated that soil fungi can be considered to show stages of loss of competitive saprophytic ability and can be grouped accordingly. His group of "root-infecting fungi" includes specialised parasites and mycorrhizal fungi. These he believes to be inhabitants of root surfaces and tissues owing to their inability to compete with saprophytic soil organisms for essential nutrients.

The Ecological Status of Mycorrhizal Fungi

There is a considerable amount of evidence that Garrett's view of the ecological status of most mycorrhizal fungi is tenable. *Rhizophagus*, the causal organism of the widespread vesicular-arbuscular type of mycorrhiza, has not yet been isolated into pure culture with any certainty. Harley (6) has tabulated the results of many workers who have attempted to isolate it. He pointed out that the most that has been achieved is cross-inoculation between fragments of infected host tissue and uninfected host in culture [Magrou (7); Stahl (8)]. This organism appears to be almost restricted to living host tissue but there is a possibility that it may also exist upon dead plant material in some stages of decay because newly formed roots are initially infected from the soil.

Many endophytes of the Orchidaceae are well known in culture and rela-

[1] The survey of the literature pertaining to this review was concluded in December, 1951.

tively easy to grow, few having specialised demands. Their distribution in the soil and their activity there is almost unknown except in such cases as *Armillaria mellea*. This fungus is a rather specialised parasite which does not spread as mycelium in the soil but passes from one food base to another in the form of rhizomorphs. Its colonisation of food bases depends upon their nature and particularly upon the presence of easily hydrolysed polysaccharides in them [Leach (9, 10)]. The species of *Rhizoctonia* which form mycorrhiza with many orchids appear, from the frequency with which they have been isolated, to be widely distributed and not necessarily closely specific to particular species of orchids nor to hosts growing in particular habitats [Curtis (11)]. Little is known of their ecological status in the soil except for the observation that the symbiont of *Goodyera repens* may be isolated from freshly fallen, but not decayed, litter in conifer plantations [Downie (12)]. This might of course suggest that it has the ability to colonise senescent plant tissue and yet is unable to withstand the metabolic products of or competition from later colonists.

Little of value is known concerning the endophytes of other plants with endotrophic mycorrhiza. Boursnell's (13) recent work on the Cistaceae suggests that the endophyte of *Helianthemum chamaecistus* is seed-borne and that the infection is systemic and cyclic in a manner similar to that described by Rayner (14) for the Ericaceae.

The ecological status of the Basidiomycetes which are associated with coniferous and other forest trees in the formation of ectotrophic mycorrhiza requires more detailed consideration. Some knowledge of the physiology of many of these has been obtained by pure culture studies. Melin (15) long ago showed that these fungi were capable of producing extensive mycelia only in cultures containing simple carbohydrates. Recently (16) he has further suggested from histological observation that, since they invade the middle lamellae of the root cortex, it is probable that pectins may also serve as carbon sources for them. This has been proved to be true of *Boletus elegans*, a mycorrhizal fungus of Larch, which will also hydrolyse and utilise starch [How (17)]. The possibility that cellulytic enzymes are developed by some species will be considered later.

Melin and his associates (18 to 21) and Fries (22) have also demonstrated that many of these fungi depend absolutely upon, or are greatly stimulated by, external supplies of thiamin and sometimes of biotin. Stimulation of growth by root extracts and exudates, sporophore extracts and litter extracts in dilute solution have been observed by Melin (23) and How (24). Strong solutions of certain humus and litter extracts caused a decrease in growth rate. Such extracts in strong or weak solution have little effect on the lignin and cellulose destroying Basidiomycetes of soil horizons. There are also indications elsewhere that certain mycorrhizal fungi are stimulated in growth by the addition of amino acids to their culture solution [Melin & Norkrans (25); Melin & Mikola (26); Mikola (27); Norkrans (28)]. These rather specialised demands, together with the ease with which such fungi are

overgrown by saprophytes in soil cultures, suggest that their mycelia must be restricted in habitat in the soil.

Björkman (29, 30) and Melin (16) have pointed out that the apparent physiological specialisation of these fungi provides a rationale for their luxuriant growth on root surfaces, for here, in root excretion and in the decay of external tissues, substances essential to them may be released into the soil in sufficient quantities. Harley (3) collected evidence from published results to show that sugars, amino acids, vitamins, and other substances are released into the root region of many plants. He indicated that their presence there had been used as an explanation of the increase in numbers of various kinds of organisms in the rhizosphere including mycorrhiza-formers. The known properties of mycorrhiza-formers can therefore provide, at best, only a partial explanation of their distribution and activity in the root region. Indeed the physiology of these Basidiomycetes, so far as it is known, only distinguishes them from unspecialised soil saprophytes in an imperfect way. Certain species such as *Cenococcum graniforme* (*Mycelium radicis nigrostrigosum* of Hatch) appear according to Mikola (27) to be somewhat intermediate between those whose carbon sources are sugars and those saprophytes breaking down cellulose and lignin. Again, Norkrans (28) has demonstrated the presence of adaptive cellulytic enzymes in *Tricholoma vaccinum* and has suggested on the basis of the effect of initial doses of glucose that other adaptive systems breaking down polysaccharides may be developed by other species of the same genus. In his work on the occurrence of polyphenol oxidases in soil Basidiomycetes Lindeberg (31) also found that although most of the mycorrhiza fungi which he examined lacked these enzymes, *Boletus subtomentosus*, a mycorrhiza fungus of *Pinus montana*, [Modess (32)] and *Lactarius deliciosus*, a mycorrhizal fungus of *P. sylvestris* and *Picea abies*, produced them in quantity and a few others produced traces. The occurrence of such enzymes is often associated with an ability to break down cellulose and lignin.

There is much ignorance concerning the distribution of the mycelium of mycorrhizal fungi away from the host trees. In natural surroundings their presence is only detectable with certainty in two ways: by the presence of their reproductive bodies or by the presence of mycorrhizal roots. A large number of them, according to Modess (32), are capable of forming mycorrhizas with several host species, but the full range of hosts is not known with certainty for any one. Hence the absence of a suitable host from a given area cannot be ensured. Nevertheless there is evidence from the planting of trees in treeless areas that dormant or sterile mycelia existing in such places have been stimulated to form mycorrhizas and even to fruit by the planting of suitable hosts. This occurred in the experiments of Rayner and Neilson Jones (33) on heath soil in England, in those of McComb (34) on prairie soils and in those of Rosendahl & Wilde (35), on sand dunes and cut-over areas. Certain species seem to be able to persist in a relatively active state where no trees grow. This has been shown by Mikola (27) to be true of

Cenococcum graniforme, and her observations confirm those of Ferdinandsen & Winge (36) who found its mycelia and sclerotia in heath and mountain soil. *Boletus subtomentosus*, mentioned above as somewhat atypical amongst mycorrhiza-formers, behaved in Romell's trenching experiments in a manner which suggests that it, too, is capable of some vegetative and reproductive vigour as a free-living saprophyte [Mikola (27), Norkrans (28)]. In these experiments [see Romell (37)], when active roots were prevented by trenching from colonising an area, fruit bodies of almost all mycorrhiza-formers failed to develop there. The mycorrhiza fungi mostly seem to flourish and fruit only in the presence of active host roots.

The persistence and spread in the soil of mycelia lacking competitive saprophytic ability is a problem which has attracted the attention of many plant pathologists. Much can be learned from the results of their observations. It seems possible that mycorrhizal fungi are no more restricted in the root region to the actual mycorrhizal tips than are many root pathogens to actual lesions. It is likely that hyphae ramify far from infected sites along the surfaces of the roots. This impression is gained by examining the distribution of black mycelia, which include *Cenococcum graniforme*, on the root system of beech seedlings during the initial stages of mycorrhizal infection. If this is true, the spread of the fungus may well be effected by root contacts. Persistence in the absence of living roots may extend over many years as has already been suggested above. Such cases may find their parallel in the observations of Rishbeth (38, 39, 40) on the parasite *Fomes annosus*. This fungus, which causes serious damage both as a butt-rot and as a root pathogen of conifers, does not grow freely in the soil nor does it spread vigorously upon unsterilised plant remains. Its competitive saprophytic ability is so limited that its persistence on a given site depends upon the presence of old woody tissue in the soil where it may remain in a somewhat inactive state for as many as thirty years. Its survival depends upon the rate of decomposition of the material and upon the presence of conditions which discourage the spread of competitors which would gradually replace it. In large stumps and in deeply sunken roots it persists longest. *Trichoderma viride* is an especially vigorous competitor or antagonist of *Fomes* and on acid soils where this fungus is most active, *Fomes* is soonest eliminated. On alkaline soils, less suitable for *Trichoderma*, *Fomes* continues to exist in old wood for the longest period. Moreover, *Fomes* is able to spread along the surface of unsterilised roots from alkaline soil but not along those from acid soils.

The sensitivity of *F. annosus* to the activities of soil fungi has also been observed byBjörkman (41) who studied its reaction to antibiotics produced by the fungi found in those soils where it was encountered as a pathogen. Similar examples are known of parasites of soft rather than woody tissues which resemble *F. annosus* in their reactions. *Ophiobolus graminis* persists apart from living hosts in those conditions where high rates of decomposition of organic residues do not take place, that is where the activity of its competitors is least [Garrett (42, 43, 44); Fellows, (45)].

It seems possible that *Polystictus circinatus*, which according to Gosselin (46) causes both mycorrhiza formation and butt-rot of spruce, might behave like *F. annosus*. Recent work by Dimbleby (47, 48) on birch growing on heather moors in England has indicated, too, that such a method of persistence may be more common in mycorrhizal fungi than has been previously suspected. Dimbleby observed that on *Calluna* moors regeneration of birch was relatively difficult. The young seedlings were distributed in definite patterns which were clearly correlated with the distribution of old stumps and their roots in the soil. The presence of these stumps was in most cases unsuspected at first because the land had been under heather with only very sparse trees for a great number of years. So conspicuous was the grouping of birch seedlings that they often appeared to be arranged in lines radiating from a centre. Excavation showed that the roots of all the healthy seedlings had penetrated into decaying woody tissue in the soil and had there developed abundant branched mycorrhizal laterals. The seedlings whose roots failed to make contact with decaying wood in the soil were usually very unhealthy and short-lived and formed few mycorrhizal roots. Dimbleby's observations suggest a direct parallel with the work of Risbeth because the mycorrhizas developed only when the roots came in contact with woody material in which mycorrhizal fungi might be assumed to have persisted. The nature of these mycorrhizal fungi was indicated by the observation of fruit bodies of *Boletus scaber* and *Amanita muscaria* around those birches which had become satisfactorily established. These two fungi are known to be potential mycorrhiza-formers with birch [Modess (32)].

The observations of MacDougal & Dufrenoy (49 to 53), although not yet satisfactorily described in some particulars, may indicate a persistence of certain mycorrhizal fungi of pine for a period of 28 months upon roots detached from the parent tree in the soil. The fact that such roots remain alive and grow for some time is not necessarily a matter for great surprise, especially if they consisted in part of secondarily thickened axes or axes containing food reserves. Detached roots of other species of tree containing stored nutrients have been observed to live many months in tropical soils. Leach (10) has shown that these may be capable of carrying pathogens such as *Armillaria mellea* or of becoming infected and of serving as a source of disease to susceptible hosts whose roots come in contact with them at a later date.

All this work helps to explain the impression gained of the patchy development of mycorrhizas in some woodland areas. In beech woodland the roots colonising decayed wood deep in the soil below the humus layers often develop mycorrhizal laterals in quantity. This is in striking contrast to the development in the surrounding mineral soil. The reason may be that the mycelia persist only in decaying woody tissues or that infection is encouraged by the particular conditions of growth or by the absence of competitors in locations such as these.

All through the foregoing account competition for essential substances

has been particularly emphasised as a factor which might determine the occurrence and distribution of mycorrhizal fungi in the soil. The possibility of the existence of conditions or substances in the soil which inhibit or reduce the activity of mycorrhizal fungi cannot be ignored. Indeed, there is much evidence for the occurrence of them. The toxic condition of the soils of certain *Calluna* heaths in England has been described by Rayner & Neilson Jones (33). In these not only were mycorrhizal fungi susceptible to the influence of the toxins but the whole microorganism population appeared to be modified and reduced in activity. The biological origin of the toxins was conclusively demonstrated by experiments with sterile and reinoculated soil. Such an extreme condition as this may not often be encountered, yet the natural occurrence of substances influencing soil populations by differential inhibition has been described elsewhere. The inhibitory substances obtained from certain kinds of litter by Melin (23) are examples. More recently Handley (54) has extracted moist *Calluna* raw humus collected from *Calluna* heathland with distilled water and obtained solutions of about pH 3.7 which, when added to a nutrient solution and inoculated with various mycorrhizal fungi, caused a nearly total inhibition of growth of all except *Boletus scaber*. The following species were tested: *B. bovinus*, *B. variegatus*, *B. granulatus*, *B. elegans*, *B. scaber*, *Amanita muscaria*, *Rhizopogon luteus*, and *Mycelium radicis sylvestris* α. The inhibition was not due to the low pH of the extract because nutrient solutions without extract support growth at pH 3.6. Extracts were inoculated with unsterilised *Calluna* humus and considerable growth of soil fungi occurred on them. After three weeks growth the filtrate of the medium from such cultures supported the growth of all the mycorrhizal fungi tested at pH 3.65. These experiments demonstrate that a soluble inhibitor which influences the growth of numerous mycorrhizal fungi is obtainable from *Calluna* soils of the same nature and from the same district as those observed by Dimbleby. They also demonstrate that the inhibitor may be destroyed by the activity of soil fungi. One is led to suppose that the discontinuous distribution of mycorrhizal development observed by Dimbleby might be explicable in terms either of the absence of the inhibitor in decaying stumps in the soil or of the presence in such locations of microorganisms active in the destruction of it.

From the same locality Leyton (55) has obtained interesting results by removing living *Calluna* and *Calluna* humus from the surface of the soil in plantations of Norway spruce and Corsican pine. Stands consisting of alternate lines of these two trees had grown in a stunted and unhealthy manner for 15 years. In June, 1949, sample blocks were treated in two ways: removal of *Calluna* and application of graded doses of phosphatic manure. In September, 1949, after four months, the trees on those blocks from which *Calluna* had been removed had produced along the lines of the trees a crop of sporophores mainly of *Boletus* species. (*Boletus luteus* was the most common). The sporophores were virtually absent from the uncleared areas, few were found on clear areas receiving no phosphates, and they were abundant on all those areas receiving phosphate manure, irrespective of the quantity.

Subsequent observations have shown that the trees in the cleared areas have grown at greater rates and that their needles have become of deeper green and of higher nitrogen content than those of the control areas. These results again suggest that the presence of growing *Calluna* and *Calluna* humus so affects the development of mycorrhizal fungi that they neither infect the host roots nor fruit in a normal manner. The results also show how tree growth, mycorrhizal development, and fungal vigour may be interrelated in so complex a manner that chains of causation cannot be determined.

Further investigation is greatly needed concerning the ecology of the mycelia of the mycorrhizal fungi. The evidence available at present suggests that the majority are unable by themselves to co-exist saprophytically with other soil fungi and that they can develop completely and fruit only in contact with living host roots. If this be true, then such stimulative effect as they may have on their hosts, if it is not due to purely internal activity, must be dependent initially upon materials that they can obtain from the roots. Such materials might so affect their vigour as to enable them to compete with other soil fungi. Indeed, the evidence is somewhat against the views of Burges (56) who believed that infection is unimportant and that the activity of the fungi in the soil is all important to the nutrition of the host. At present it is possible to imagine that the host root, after infection, acts as a center from which the mycorrhizal fungi exploit the humus layers in competition with free-living saprophytes. This is the opinion of Björkman (30) and it will be further discussed in a later section.

Infection of Roots by Mycorrhizal Fungi

The conditions necessary for infection of host roots by mycorrhizal fungi have been studied in the case of ectotrophic mycorrhizas, but among endotrophic forms, those due to *Armillaria mellea* alone can receive comment at present. In the latter cases the attack follows, as far as can be seen from the work of Kusano (57), Hamada (58), and Burgeff (59), the sequence outlined by Garrett (60) for root-infecting pathogens. *Armillaria* attacks the orchids *Gastrodia* and *Galeola* from a center or food base in decaying wood. The fungus develops sufficient "infective power" by absorption of food from the wood to overcome host resistance in the orchid and to colonise the tissues. Since little is known of the life of other mycorrhizal fungi in the soil, no such sequence can be visualised. Nevertheless the work of Hatch (61), Björkman (29) and others has provided some knowledge of the effect of external conditions on the susceptibility of hosts in the case of conifers. The results of this work are summarised in the papers of Harley (3) and Björkman (30) and need not be repeated in detail. Since Björkman's work has been much misinterpreted and misunderstood, some reference to it must be made. The accounts of his views given by Schmidt (62) and Clark (63) are erroneous and misleading and may be due, so Björkman (30) believes, to difficulties in the Swedish text of a review of his work and in its English translation (64). He has restated his views clearly in the paper quoted.

There are briefly two sets of factors which markedly affect mycorrhizal

development: factors which affect the synthesis of carbohydrate in the plant and factors which affect nutrient availability in the soil. The initiation of infection usually only occurs when free sugars or available carbohydrates are present in the root tissues. In low light intensity, in plants with reduced leaf, area, and in partly ringed plants, mycorrhizas are generally poor in development. They are also poorly developed in plants heavily manured with essential minerals such as nitrogen, phosphorus and perhaps potassium. Excessive mineral manuring is believed to decrease mycorrhizal development owing to increased synthesis of living matter within the plant and consequent reduction of free sugars or other carbohydrates.

As Harley (3) has pointed out, this hypothesis fits remarkably well with a large number of varied results of experiments upon the initiation of infection. Björkman (30) later showed that in heavily manured plot and pot experiments coniferous seedling failed to develop mycorrhizal roots at first, but after a period of years, these were formed as mineral deficiency developed. Björkman believes that the formation of mycorrhizal roots occurs as the result of a direct response of the fungus to available sugar in the roots. This may be true, but it remains probable that some substance which stimulates the fungus must be released into the soil by the roots. This external stimulus may be carbohydrate, but a study of the variations of root excretions or losses in quantity or in nature has not been made under different nutritional conditions. The stimulation of seedling growth which has been observed to occur after infection is not believed by Björkman to follow the removal of sugars by the fungus. This remarkable hypothesis, which has been attributed to him, appears to have arisen by a misunderstanding of Swedish.

Infection and the Structure of the Host Plant

The most interesting recent results upon the effect of mycorrhizal infection upon the structure of the host relate to ectotrophic mycorrhizas. Clowes (65, 66) has made a detailed histological study of the roots of *Fagus sylvatica*. He has demonstrated that the essential plan of construction and the behaviour of the meristems and histogens is similar in infected and uninfected roots. Their difference in appearance is not due to hypertrophy (expansion of cell volume) or to hyperplasia (cell proliferation) but is due partly to the change in the shape of cells in the cortex. In infected roots some of the cortical cells show a reduced longitudinal expansion and an increased transverse expansion. This, together with the presence of the sheath, is responsible for the swollen appearance of the mycorrhizas. He found evidence that growth in length might be reduced not only by the reduction in cell elongation but also by reduced meristematic activity. An analysis of the distances behind the apical meristem at which stages of maturation occurred showed striking differences from uninfected roots. By comparing roots of equivalent diameter Clowes showed that vacuolation of the cortex, tannin impregnation of the endodermis and lignification of the protoxylem all occurred nearer the apex in infected roots. In addition, he observed that the sculptur-

ing of the lignified walls of the protoxylem was usually pitted in mycorrhizal roots whereas it was annular or annular-spiral in uninfected roots. Clowes pointed out that the sculpturing of the protoxylem is often thought to be related to the rate of growth of the apex, and that this is evidence for the view that the growth rate of mycorrhizal axes was reduced.

The importance of these observations lies in the way they contrast in certain respects with the results of others. Hatch (61) has summarised the effects which mycorrhizal development is believed to have on pine roots. These include continuous growth and branching and also delayed development (i.e. development farther from the apex) of the mature endodermis. These points have been considered of importance in relation to the theory of mycorrhizal action.

A further important advance has been due to Slankis (67 to 72) who has used excised pine roots growing in culture. His first experiments (67, 68) described the methods used and demonstrated that glucose and sucrose were effective sources of carbon for growth, sucrose being the better. He next (69) showed that species of *Boletus*, especially *B. variegatus*, when placed in the culture solution caused the development of dichotomous short laterals superficially similar to mycorrhizas even in positions on the long roots far from the hyphae and mycelium of the fungus. Filtrates of cultures of *B. variegatus* which might be expected to contain exudates from or by-products of the metabolism of its mycelium exerted a similar effect on the roots if introduced into the culture medium. Hetero-auxin (β-indole-acetic acid) and other compounds having auxin-like properties were shown to have an effect similar to *Boletus* filtrates (70, 72). In low concentrations (0.001 M μg to 1.0 μg per 20 ml. of β-indole-acetic acid) the formation and growth of long roots was especially stimulated, but at higher concentrations the number of short branches was increased while long root development was inhibited. In particular, these short branches developed dichotomous forking and resembled mycorrhizas to a remarkable degree. In some conditions repeated forking was observed, giving rise to lateral systems similar to those of mycorrhizas found in soils where considerable infection develops. Where dichotomy was encouraged in the short roots, inhibition of root-hair formation occurred.

These results are particularly important because they suggest a way by which the fungi may alter the appearance and the sequence of maturation of the roots of their host. Slankis has not yet provided information concerning the nature of the histological changes in roots modified by extracts of fungi and by phytohormones. These should be of very great interest. He observed (72) that one of the effects of his treatments was to increase the total root surface by an increase of branching and dichotomy. This tends to confirm one of the points made by Hatch (61) that the total root surface which is capable of absorbing soil nutrients is greater in infected than in uninfected pine seedlings. It also tends to suggest that the criticism of this view based on an examination of root/shoot ratios, which was put forward by Harley (3) is not necessarily firmly founded.

The production of swollen rootlets in the soil has sometimes been recorded to occur before or in the absence of mycorrhizal development. McComb & Griffith (73) observed that in uninoculated experimental plots of spruce, hypertrophied short roots developed. These were devoid of fungal mantle and appeared uninfected. Their number increased with increasing phosphorus manuring. A somewhat similar phenonemon has been noted in *Fagus sylvatica* by Wilson (74, 75). During the early growth of beech seedlings on acid soil the sub-lateral roots passed through a stage of increase in diameter which was associated with a reduction in size of the meristem and often with hypertrophy of the cortical cells. In unsterilised soils these modified rootlets were first colonised by mycorrhizal fungi on their surface and later many developed into typical mycorrhizas. Further observations showed that these modifications of the rootlets are not necessarily only a stage in the development of mycorrhizas, nor are they occasioned by the activities of microorganisms for they occur in sterilised soil in which no mycorrhizas are formed. There was some evidence that the hypertrophied laterals might be more short-lived than mycorrhizal laterals. It is interesting to observe in connection with the development of swollen laterals, in aseptic conditions, that Slankis (71) has observed these in his cultures of pine roots at temperatures away from their optimum.

Clowes (66) has observed the effect of heteroauxins and of colchicine upon attached roots of seedling beech. He pointed out that those concentrations which reduce the rate of growth in length bring about radial and tangential enlargement of the cortical cells and a reduction of their elongation. In colchicine tumors (formed in 0.01 to 0.02 per cent aqueous solutions) differentiation of the cells close to the apex occurred, and pitted xylem elements, rather than spiral or annular elements, developed nearest the apex.

This aspect of work on the effects of mycorrhizal and other organisms of the rhizosphere upon root structure has only just begun. The observation that many factors which reduce elongation have similar effects upon the internal structure of the root is important and note should also be taken of Smith & Kersten's work with soft x-rays in this connection (76). The possibility that internal factors may lead to swelling or hypertrophy of lateral roots must be kept in mind, for these are suggested by the work of McComb & Griffith (73) and Wilson (74, 75). Internal vitamin deficiency is suggested as possibly important by Clowes (66) and has, of course, been demonstrated as an important factor in the causation of the swelling of the cells of orchid seeds when they become glutted with starch in the absence of fungi [Schaffstein (77, 78)]. It is too early yet to expand this thesis further.

Mycorrhizal Roots as Nutrient-Absorbing Organs

There can be no reasonable doubt that mycorrhizal roots are nutrient-absorbing organs for the very reason that host plants remain healthy and grow even when almost all their unthickened roots are converted into mycorrhizas. This is especially obvious in the case of ectotrophic mycorrhizas where

the vast majority of the feeding roots are not only infected but also covered completely with a fungal pseudo-parenchyma. Clowes (66) has shown that even some of the long leading roots of *Fagus sylvatica* may be ensheathed almost completely by fungal tissue even when typical intercellular infection of the cortex does not develop. In many instances the evidence is not conclusive that host plants possessing mycorrhizas grow faster or are better supplied with nutrient than those not equipped in this way. This is particularly true of some kinds of endotrophic mycorrhizas such as the vesicular-arbuscular infections. In other cases where such doubt exists there is evidence that a biological stimulus is needed for seed germination and early growth of seedlings. This seems to be true of Ericaceae and Cistaceae, amongst others [Harley (6)]. It has been fairly satisfactorily demonstrated for the Orchidaceae and for many of the forest trees in the Cupuliferae and Pinaceae that mycorrhizal plants are enabled to absorb substances from the soil better than their uninfected equivalents in some conditions. It is highly desirable that these results should be further elaborated by an elucidation of the mechanism of the absorption of materials by mycorrhizas.

Harley (6, 79) has attempted to summarise the conditions, at least one of which must be fulfilled, if increased absorption by mycorrhizal roots is to occur. The following are relevant here: (*a*) The fungus might fix atmospheric nitrogen in the tissues of its host. (*b*) A secretion of enzymes or other substances by the fungus or by the mycorrhiza might increase local availability of materials in the soil. (*c*) A purely internal activity of the fungus might produce essential accessories available to the host. (*d*) Infection might result in a change in area or nature of the absorbing surface so that absorption is facilitated. (*e*) Hyphal connections between the mycorrhiza and the soil might act as organs of conduction of material into the host tissue.

(*a*) Nitrogen fixation has been eliminated as a possibility except perhaps in *Ericaceae* infected with *Phoma* [compare Rayner (14) and Freisleben (80, 81)] because none of the fungi which have been shown to form mycorrhizas with other plants seem to be capable of fixing significant amounts of atmospheric nitrogen.

(*b*) The secretion of enzymes by infected roots was suggested by Ziegenspeck (82) for the Orchidaceae, but his results must remain a little suspect until they are repeated under aseptic conditions. A local external increase of essential materials is required according to the theory of the action of mycorrhizas by Burges (56). The results of Mitchell, Finn & Rosendahl (83) showed no such increase, but this, as Harley (3) has pointed out, does not in itself dispose of Burges' hypothesis. This hypothesis has already been criticised above on other grounds. Nevertheless, it may be still tenable in the light of such work as that of Gerretsen (84) on the effect of rhizosphere bacteria upon the availability of phosphates in the root region.

(*c*) The provision by the fungus of essential accessories may perhaps be important in many cases where some biological stimulation of germination and early growth is essential to successful development. It is held to apply

to many Orchidaceae, to Ericaceae, and to plants of other families. For the extensive literature on this subject reference may be made to Harley (6). This activity of the fungi, where it exists, only affects very indirectly the absorption of materials from the soil.

(*d*) and (*e*) The increase of absorbing area of the root surface and the presence of outgoing hyphae have been made essential constituent parts of the well-known hypothesis of Hatch (61) concerning absorption of materials by ectotrophic mycorrhizas. The study of the degree to which mycorrhizal roots are connected by hyphae to the soil has received remarkably little attention considering its potential importance to the physiology of absorption by mycorrhizas. If substances are passed in significant quantities into the host through such hyphae, several additional conditions must be fulfilled. Firstly, the fungus must have absorbing organs in the soil through which materials are absorbed in excess of its own requirements. Secondly, this absorption must take place in competition with other soil organisms. Thirdly, enough connections between the absorbing mycelium and the mycorrhizal root must exist to carry the required quantities of material into the plants. Lastly, a mechanism must exist whereby the materials absorbed into the fungal tissue are transferred in significant quantities to the host.

Knowledge of the numbers of hyphal connections between mycorrhizal roots and the soil is surprisingly sketchy. Burgeff (96) has attempted to estimate the numbers of hyphae connecting the roots of the orchid *Platanthera chlorantha* to the soil. A single plant with five roots had a calculated number of 32,000 outgoing hyphae. In Burgeff's opinion this was a sufficient number to provide an adequate path for the absorption of materials into the orchid host, and he pointed to other evidence in support of it. For instance, in certain artificially inoculated cultures, seeds on the glass side of the culture vessel appeared to grow and absorb nutrient when attached to the culture medium by hyphae only. The hyphal connections between ectotrophic mycorrhizas and their substrates appear to be extremely varied in number. Certain fungi, such as *Cenococcum graniforme*, normally form mycorrhizas with extensive development of outgoing hyphae [Mikola (27, 85)]. Newly infected laterals in woodland soil and more especially in culture vessels, may also appear to possess many connections. Adult mycorrhizas in the humus and leaf litter horizons of forest soils are more variable and often appear macroscopically to be smooth and to lack outgoing hyphae. Indeed, photographs and drawings of microscopic preparations of these often show a smooth surface. Some impression may be gained of their variability from Björkman's paper (29) and from examination of photographs of mycorrhizal roots in the published plates of such a work as that of Hatch (61). Inspection of mycorrhizal roots of beech from the litter and humus layers shows them to be smooth at first sight. Careful observation indicates that, except perhaps very near the tip, the sheath is usually tightly adpressed to the plant debris and to soil particles so that these are pulled clear only with some difficulty. In some cases the sheath tissue is connected to the soil by strands

of hyphae and may be locally somewhat winged owing to aggregations of the hyphal strands. The connections with the substrate do not appear to be uniformly distributed. This is a subject like others concerning the activity of the fungi in the soil upon which exact knowledge is deficient, and it is one of some practical difficulty.

Recent experiments of Melin & Nilsson (86) upon the phosphorus absorption of pine seedlings, using radioactive P^{32} as a tracer, have served to show that outgoing hyphae where they occur in quantity can conduct materials to the host. In these experiments pine seedlings were grown in pure culture in sand and were inoculated by placing on the surface of the sand small dishes containing an appropriate fungus in a culture medium. In due course the fungus grew over the lips of the dish and, passing through the sand culture, caused mycorrhizas to develop on the short roots of the pines. The dishes were now supplied with a solution of phosphorus containing a considerable quantity (1 mc.) of P^{32}. Later observation with a Geiger counter showed that the radioactive material had passed not only into the roots of the seedlings but had also been conducted into the leaves. At the same time the sand in the culture medium showed some activity, and, although this might itself suggest criticism, it is quite likely that it was due to fungal hyphae in the sand. At all events there is a high probability that the hyphae conducted phosphates into the mycorrhizas and that this was passed on to the host tissues and translocated to the leaves.

These experiments seem to demonstrate three important points: the absorption of minerals by the fungal mycelium at a distance from the host, the conduction of these materials along hyphae, and the loss of them into the host tissue. It is important to note that they give no information about the rate of these processes, and that information on their rates would be especially desirable for consideration of the mechanism of absorption by mycorrhizal seedlings.

It has been pointed out by Harley & McCready (87) that in considering the passage of salts into plants with ectotrophic mycorrhizas there are four important phases, absorption and conduction of material by the fungal hyphae, absorption and accumulation of material by the fungal sheath, release of material by the sheath and Hartig net into the host tissues, and translocation within the host. Absorption and passage of salts through the sheath tissues themselves has always been considered an important route and in one experiment with mature beech in a woodland site, Harley & McCready (87) have shown that phosphorus may enter and pass up a plant by this way. It is this route which is mainly in mind when the view is expressed, as by Hatch (61), that the surface of the roots of mycorrhizal seedlings exceeds in area that of non-mycorrhizal seedlings. Clowes' view that growth by the activity of the apical meristem and by cell elongation are both reduced in beech mycorrhiza has already been noted. In this case an increase of surface area of the root system could not be dependent on the growth of individual axes in the length. Nevertheless, increase of diameter by transverse cell

expansion and by the presence of the fungal sheath might offset the reduction in area caused by reduced growth in length to an unknown extent. Another reaction which might compensate for reduced growth in length of the individual axes is increase of the frequency of branching. There is general agreement that increased branching occurs in mycorrhizal plants but it is extremely difficult to evaluate it. If it is true that the absorbing surface of mycorrhizal plants is greater than that of non-mycorrhizal plants, and this appears to be possible, then a comparison of the rates of salt accumulation in equal areas of roots of the two kinds appears desirable.

Routien & Dawson (88) have made the assumption, for the study of the physiological problems of root systems, that the linear extent of the long roots of *Pinus echinata* was the same in mycorrhizal and non-mycorrhizal plants. Harley & McCready (89) have pointed out that such assumptions do not appear to be satisfactory as generalisations. The considerable changes in relative weights and in the form of seedlings associated with mycorrhizal infection may often vitiate such assumptions. In experimental work comparing the physiological properties of mycorrhizal and non-mycorrhizal roots, it therefore appears advisable to use a simpler standard. The comparison of the properties of equivalent apices sampled in some adequate manner upon the basis of area or weight seems the most satisfactory first approximation. The results of experiments with such samples give information concerning the nature of the surfaces and the properties of the constitutent cells of the mycorrhizal and non-mycorrhizal roots, leaving questions of the total size of the surfaces of the roots of the whole plant aside. Such an approach to the problem of salt absorption by mycorrhizal plants falls in line with the recent work on the physiology of salt absorption by roots and allows similar methods to be employed. Work on these lines has already yielded some results.

The Absorption of Minerals by Mycorrhizal Roots

Kramer & Wilbur (90) have examined the absorption of phosphorus by excised roots of *Pinus taeda* and *P. resinosa* using P^{32} as a tracer. With excised branching systems upon which infected and uninfected apices were present they obtained visual estimates of the accumulation of radioactive material by photographic means. Those parts of the root system equipped with fungal sheaths were shown to absorb phosphorus at a greater rate than uninfected roots from solutions containing very low phosphate concentrations. These results were confirmed by estimates of the radioactivity of unit lengths of uninfected and infected roots with a Geiger counter. Similar results were obtained by Harley & McCready (89) using the root systems of *Fagus sylvatica*. In this case the results of counts on the Geiger counter were expressed in terms of radioactivity per unit area of root surface or per unit fresh weight of material. Under the conditions used, at pH 5.5, the rate of accumulation in infected roots was on an average about four times that of

uninfected roots on an area basis and in some estimates up to ten times that of uninfected roots. Over a range of pH from 3.4 to 6.3 in phthalate buffer the absorption by mycorrhizal roots exceeded that of non-mycorrhizal roots at all pH values both on a fresh weight and on an area basis.

Kramer & Wilbur (90) observed that pseudo-mycorrhizas of pines also accumulated phosphorus at greater rates than uninfected roots. Harley & McCready (89) showed that roots upon which only local patches of sheath or incomplete sheaths occurred showed higher rates of phosphorus absorbtion than uninfected roots. Photographic film which had been exposed in the dark to transverse sections of mycorrhizal roots was darkened in an annular region opposite the fungal sheath. All these observations suggested that the main site of accumulation of phosphorus in mycorrhizas might be the fungal tissue rather than the host tissue.

In later papers Kramer (91) and Harley & McCready (92) have carried these observations further. It has been shown that respiratory inhibitors and reduced oxygen tensions decrease the rate of phosphorus absorption into the tissues. This process is therefore dependent upon metabolic activity. Absorption continues at an approximately constant rate from low concentrations of phosphorus over a period of several hours. Its magnitude is also dependent upon the phosphorus concentration, and it increases in the case of beech mycorrhizas up to concentrations of at least 32 millimoles of potassium phosphate at pH 5.5. During absorption the internal concentration of phosphorus calculated in terms of phosphorus per unit volume of root tissue may rise to a level many times that of the applied concentration.

Harley & McCready (87) have shown that it is possible to separate the fungal sheath from the core of mycorrhizal roots of the beech and that from well-aerated solutions at pH 5.5, containing phosphate concentrations less than 1 mM, approximately 90 per cent of the phosphate absorbed was retained in the fungal sheath.

The fungal tissue comprises some 20 to 30 per cent of the total volume and about 39 per cent of the total dry weight of the mycorrhizal apices of beech. Hence the accumulation in the sheath of intact tips far exceeded that in the cores on a weight or volume basis. Both cores and sheaths exposed separately to phosphate solution absorbed phosphorus more rapidly than when associated in intact tips, but the avidity of the sheath exceeded that of the core in all conditions studied. In the conditions used, the fungal sheath, therefore, prevents the core from accumulating phosphorus at its maximum possible rate. Certain external factors increase the proportion of the absorbed phosphorus which enters the core. The presence of respiratory inhibitors or the absence of oxygen reduce total uptake very greatly but increase the proportion of phosphorus entering the core. High phosphorus concentrations increase the total rate of accumulation and the proportion entering the core.

These experimental results, although interesting in themselves, suggest

many further problems, and they are not yet very useful in helping to explain the results of observations upon whole plants. No evidence is yet available concerning the actual route or mechanism by which salts pass through the fungal sheath into the host. This might be by diffusion through the cell walls and intercellular spaces of the sheath, or by way of the living fungal sheath cells.

In the ectotrophic mycorrhizas of pine and beech no digestion of fungal hyphae normally occurs in the tissues. The movement of phosphorus into the host in Melin & Nilsson's experiment (86) must have been from living fungal sheath cells into living host cells. It is important to confirm that this is also the route of absorption into the cores of excised mycorrhizas before the results concerning the effect of external conditions on the rate of absorption can be used to interpret results from whole plants.

Even in examples of endotrophic mycorrhizas in which digestion in the host cells is a usual occurrence, this is not the only method whereby material may pass from fungus to host. Harley (6) has suggested the possibility that digestion, by virtue of its conspicuousness, may have received more attention than it deserves. Movement of material from the cells of a host into the cells of a parasite does not necessarily depend upon digestion, and many parasites which kill their hosts in advance of their hyphae secrete substances into the tissues. The movement of both organic and inorganic materials into and out of the living hyphae of fungi in pure culture occurs as a normal process [Foster (93)]. Hence there is no need to believe that digestion is necessary to explain a gain of material by the host from the fungus.

Although work with excised roots has, as yet, yielded little information on mechanisms, there is one hypothesis that can be formulated and examined which arises from it. Harley (94) has pointed out that the period of life of mycorrhizal roots in the soil is relatively short. They are essentially deciduous and are said to be active for periods of up to about three years. During life they appear, from the experimental work, to be able to accumulate considerable quantities of phosphorus in their tissues and although this may be mainly into the fungal tissues, it does represent an accumulation in the root region of the host. On death of the mycorrhiza, such material may well be released into the root region in soluble form. Here, perhaps, is a mechanism whereby the quantity of phosphorus in the rooting region is increased by the production of decaying organic matter of high phosphorus content. Although this suggestion has certain attractive points, it does not explain satisfactorily much of the work with young seedlings. The stimulation of the growth of seedlings by mycorrhizal infection seems to develop too rapidly after infection for such a mechanism to operate. The mechanism could only be invoked in the case of mature trees. Moreover there is, evidence in the work of Routien & Dawson (88) that increased absorption by infected seedlings occurs directly under cultural conditions from artificial colloidal soil. These workers suggested that the mechanism of increased absorption de-

pended upon the increased respiration rate which could be observed in a comparison between equal lengths of mycorrhizal and non-mycorrhizal long roots bearing equivalent numbers of primary laterals. The increased respiration was held to result in increased availability of hydrogen ions which could replace the cations on the colloidal particles of the substrate. It is perhaps doubtful if this comparison of respiration rates is a satisfactory one. Increased uptake of salts by infected roots on a weight or area basis has been observed, whereas in these experiments the quantities of plant tissue were vastly different in the two kinds of sample. These matters, therefore, need further careful attention, and comparisons on a more equivalent basis would be very valuable.

The aim of this article is not to cover completely the work on mycorrhizas published in any stated period of years. It has aimed, rather, at considering those parts of the subject which do not appear to have been adequately discussed in recent reviews. An extensive account of recent mycorrhiza literature is to be found in the monograph by Kelley (95).[2]

[2] The author wishes to thank those friends and colleagues who have allowed him to use the results of some of their experiments which have not yet been published.

LITERATURE CITED

1. Katznelson, H., Lochhead, A. G., and Timonin, M. I., *Botan. Rev.*, **14**, 543–87 (1948)
2. Smith, N. R., *Ann. Rev. Microbiol.*, **3**, 453–84 (1948)
3. Harley, J. L., *Biol. Revs. Cambridge Phil. Soc.*, **23**, 127–58, (1948)
4. Garrett, S. D., *New Phytologist*, **50**, 149–66 (1951)
5. Garrett, S. D., *Biol. Revs. Cambridge Phil. Soc.*, **25**, 220–51 (1950)
6. Harley, J. L., *New Phytologist*, **49**, 215–47 (1950)
7. Magrou, J., *Compt. rend.*, **203**, 887–88 (1936)
8. Stahl, M., *Planta.*, **37**, 103–48 (1949)
9. Leach, R., *Proc. Roy. Soc.* (*London*), [B]**121**, 561–73 (1937)
10. Leach, R., *Trans. Brit. Mycolog. Soc.*, **23**, 320–29 (1949)
11. Curtis, J. H., *Am. J. Botany*, **26**, 390–98 (1939)
12. Downie, D. G., *Trans. Proc. Botan. Soc. Edinburgh*, **33**, 383–90 (1943)
13. Boursnell, J. G., *Ann. Botany*, **14**, 217–43 (1950)
14. Rayner, M. C., *Mycorrhiza*, 93–112 (Cambridge Univ. Press, London, England, 286 pp., 1927)
15. Melin, E., *Untersuchungen über die Bedeutung der Baummykorrhiza* (Gustav Fischer, Jena, Germany, 152 pp., 1925)
16. Melin, E., *Trans. Brit. Mycolog. Soc.*, **30**, 92–99 (1948)
17. How, J. E., *Ann. Botany*, **4**, 135–40 (1940)
18. Melin, E., and Lindeberg, G., *Botan. Notiser*, 241–45 (1939)
19. Melin, E., and Norkrans, B., *Svensk Botan. Tid.*, **36**, 271–86 (1942)
20. Melin, E., and Nyman, B., *Arch. Mikrobiol.*, **11**, 318–28 (1940)
21. Melin, E., and Nyman, B., *Arch. Mikrobiol.*, **12**, 254–59 (1941)
22. Fries, N., *Symbolae Botan. Upsalienses*, **6**(4), 81 (1943)
23. Melin, E., *Symbolae Botan. Upsalienses*, **8**(3), 116 (1946)
24. How, J. E., *Ann. Botany*, **5**, 121–31 (1941)
25. Melin, E., and Norkrans, B., *Physiol. Plantarum*, **1**, 174–84 (1948)
26. Melin, E., and Mikola, P., *Physiol. Plantarum*, **1**, 110–13 (1948)
27. Mikola, P., *Commun. Inst. Forestales Fenniae*, **36**(3), 104, (1948)
28. Norkrans, B., *Symbolae Botan. Upsalienses*, **11**(1), 126 (1950)
29. Björkman, E., *Symbolae Botan. Upsalienses*, **6**(2), 191 (1942)
30. Björkman, E., *Svensk Botan. Tid.*, **43**, 223–62 (1949)
31. Lindeberg, G., *Physiol. Plantarum*, **1**, 196–205 (1948)
32. Modess, O., *Symbolae Botan. Upsalienses*, **6**(1), 147 (1941)
33. Rayner, M. C., and Neilson Jones, W., *Problems in Tree Nutrition*, 43–44 (Faber & Faber Ltd., London, England, 184 pp. (1944)
34. McComb, A. L., *Agric. Exp. Sta.* (*Iowa*) *Bull.*, **314**, 582–612 (1943)
35. Rosendahl, R. O., and Wilde, S. A., *Bull. Ecol. Soc. Am.*, **23**, 73–74 (1942)
36. Ferdinansen, C., and Winge, O., *Kgl. Veterinaer Landbokögsk Aasskr.*, 332–83 (1925)
37. Romell, L. G., *Svensk. Botan. Tid.*, **32**, 88–89 (1938)
38. Rishbeth, J., *Ann. Botany*, **14**, 365–83 (1950)
39. Rishbeth, J., *Ann. Botany*, **15**, 1–21 (1951)
40. Rishbeth, J., *Ann. Botany*, **15**, 221–46 (1951)
41. Björkman, E., *Physiol. Plantarum*, **2**, 1–10 (1949)
42. Garrett, S. D., *Ann. Applied Biol.*, **25**, 742–66 (1938)

43. Garrett, S. D., *Ann. Applied Biol.*, **27**, 199–204 (1940)
44. Garrett, S. D., *Ann. Applied Biol.*, **31**, 186–91 (1944)
45. Fellows, H., *J., Agr. Research*, **63**, 715–26 (1941)
46. Gosselin, R., *Farlowia*, **1**, 525–68 (1944)
47. Dimbleby, G. W., *The Ecology of some British Podsol Formations* (Doctoral thesis, Oxford University, England, 1950)
48. Dimbleby, G. W., *Plant and Soil* (In press)
49. MacDougal, D. T., and Dufrenoy, J., *Yearbook Am. Phil. Soc.*, 214–17 (1940)
50. MacDougal, D. T., and Dufrenoy, J., *Yearbook Am. Phil. Soc.*, 161–62 (1941)
51. MacDougal, D. T., and Dufrenoy, J., *Yearbook Am. Phil. Soc.*, 170–75 (1943)
52. MacDougal, D. T., and Dufrenoy, J., *Plant Physiol.*, **19**, 440–65 (1944)
53. MacDougal, D. T., and Dufrenoy, J., *Plant Physiol.*, **21**, 1–10 (1946)
54. Handley, W. R. C. (Unpublished data)
55. Leyton, L. (Unpublished data)
56. Burges, A., *New Phytologist*, **35**, 117–31 (1936)
57. Kusano, S., *J. Agric. Tokyo*, **4**, 1–66 (1911)
58. Hamada, M., *Japan. J. Botany*, **10**, 157–211 (1940)
59. Burgeff, H., *Saprophytismus und Symbiose*, 161–90 (Gustav Fischer, Jena, Germany, 249 pp., 1932)
60. Garrett, S. D., *Root Infecting Fungi* (Chronica Botanica Co., Waltham, Mass. 177 pp., 1944)
61. Hatch, A. B., *Black Rock Forest, Bull., No. 6*, 168 (1937)
62. Schmidt, E. L., *Soil Sci.*, **64**, 459–68 (1947)
63. Clark, F. E., *Advances in Agronomy*, **1**, 241–88 (1941)
64. Baldwin, H. I., *Farlowia*, **1**, 619–24 (1944)
65. Clowes, F. A. L., *New Phytologist*, **49**, 248–68 (1950)
66. Clowes, F. A. L., *New Phytologist*, **50**, 1–16 (1951)
67. Slankis, V., *Nature*, **160**, 645–46 (1947)
68. Slankis, V., *Physiol. Plantarum*, **1**, 278–89 (1948)
69. Slankis, V., *Physiol. Plantarum*, **1**, 390–400 (1948)
70. Slankis, V., *Svensk Botan. Tid.*, **43**, 603–7 (1949)
71. Slankis, V., *Physiol. Plantarum*, **2**, 131–37 (1949)
72. Slankis, V., *Symbolae Botan. Upsalienses*, **10**(3), 65 (1951)
73. McComb, A. L., and Griffith, J. E., *Plant Physiol.*, **21**, 11–17 (1946)
74. Wilson, J. W., *Microorganisms in the Rhizosphere of Beech* (Doctoral thesis, Oxford University, England, 1952)
75. Harley, J. L., *Nature*, **164**, 1041–42 (1949)
76. Smith, G. F., and Kersten, H., *Bull. Torrey Botan. Club*, **69**, 221–34 (1942)
77. Schaffstein, G., *Jahrb. wiss. Botan.*, **86**, 720–52 (1938)
78. Schaffstein, G., *Jahrb. wiss. Botan.*, **90**, 141–98 (1942)
79. Harley, J. L., *School Sci. Rev.*, **119**, 62–71 (1951)
80. Freisleben, R., *Jahrb. wiss. Botan.*, **80**, 421–56 (1934)
81. Freisleben, R., *Jahrb. wiss. Botan.*, **82**, 413–59 (1936)
82. Ziegenspeck, H., *Lebensgeschichte der Blütenpflanzen Mitteleuropas: 1.4. Orchidaceae.*, 22–24 (Kirchner, Loew & Schröter, Stuttgart, Germany, 840 pp., 1936)
83. Mitchell, H. L., Finn, R. F., and Rosendahl, R. O., *Black Rock Forest Papers*, 1 (10), 58–73 (1937)
84. Gerretsen, F. C., *Plant and Soil*, **1**, 58–81 (1948)

85. Mikola, P., *Arch. Soc. Zool. Botan. Fenn.*, **1**, 81–85 (1946)
86. Melin, E., and Nilsson, H., *Physiol. Plantarum*, **3**(1), 88–92 (1950)
87. Harley, J. L., and McCready, C. C., *New Phytologist*, **51**, 56–64 (1951)
88. Routien, J. B., and Dawson, R. F., *Plant Physiol.*, **30**, 440–51 (1943)
89. Harley, J. L., and McCready, C. C., *New Phytologist*, **49**, 388–97 (1950)
90. Kramer, P. J., and Wilbur, K. M., *Science*, **110**, 18–19 (1949)
91. Kramer, P. J., *Plant Physiol.*, **26**, 30–36 (1951)
92. Harley, J. L., and McCready, C. C., *New Phytologist* (In press)
93. Foster, J. W., *Chemical Activities of Fungi*, 148–83 (Academic Press, Inc., New York, N. Y., 645 pp., 1949)
94. Harley, J. L., *Soc. Gen. Microbiol., Symposium on Rhizosphere* (Manchester, England, Sept. 18, 1951)
95. Kelley, A. P., *Mycotrophy in Plants* (Chronica Botanica Co., Waltham, Mass., 223 pp., 1950)
96. Burgeff, H., *Samenkeimung der Orchidum*, 16–21 (Gustav Fischer, Jena, Germany, 312 pp., 1936)

ANTIBIOTICS[1]

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No new antibiotics have come into widespread use since terramycin was introduced in the spring of 1950. However, literally thousands of articles appeared during 1951, and much new information has been added concerning the antibiotics already available. Aside from purely clinical studies, interest has centered chiefly around mode of action, development of resistance, and antibiotic synergism and antagonism. In the present review, penicillin and streptomycin will be dealt with briefly, the broad spectrum antibiotics will be considered in detail, and a separate section will be devoted to important observations which have been made concerning antibiotic synergism and antagonism.

Penicillin

Mode of action.—Many of the important developments in this field were summarized by Brownlee (1) in the preceding volume of this series. More recently Umbreit (2) has written a thought provoking review in which the evidence is considered from a somewhat different point of view. It has been well established by the studies of Rowley *et al.* (3) and of Maass & Johnson (4, 5) that during growth penicillin becomes irreversibly bound to the bacterial cell. It is postulated that the cell contains a "binding component," the synthesis of which is not prevented by penicillin, but which unites with penicillin once it is formed so that its normal function is prevented (2). Evidence for the failure of this hypothetical substance to function normally has been obtained in two ways, by measuring the lack of formation of end products, and by observing the accumulation of intermediates. The work of Gale and associates in England is an example of the first approach. The glutamic acid concentrating ability of washed (resting) staphylococci was normal in the presence of penicillin, but was impaired when cells were grown in a penicillin containing medium (6, 7). This evidence may be interpreted as indicating that penicillin does not interfere with the passage of glutamate into the cell once the enzymes concerned with this process are formed, but that the antibiotic inhibits the formation of these glutamic transporting enzymes. Hotchkiss (8, 9) on the other hand observed that in the presence of penicillin, resting cells formed non-amino nitrogenous material with properties suggestive of a polypeptide. In other words, when the penicillin sensitive component was inactivated, cellular protein was not formed, but there was an abnormal accumulation of intermediates. The nature of the binding component is not known, but Umbreit believes that it may be a coenzyme of the

[1] The survey of the literature pertaining to this review was completed in March, 1952.

uridine type (10). This interpretation tends to reconcile the somewhat divergent results of Gale and of Hotchkiss, and emphasizes the possibility that they were measuring different aspects of the same phenomenon. In any event, it now seems clear that penicillin acts by causing a subtle derangement of protein synthesis, and it seems reasonable to hope that the nature of the disturbance can be determined more precisely in the near future.

Paine (11) has observed recently that bacitracin also inhibits the uptake of glutamic acid by staphylococci and has suggested that its mode of action may be similar to that of penicillin. He further noted that staphylococci made resistant to penicillin *in vitro* were also resistant to bacitracin. This is not true of strains isolated from patients; many penicillin resistant staphylococci are highly sensitive to bacitracin.

One phenomenon that must be fitted into any theory concerning the mode of action of penicillin is the observation that certain strains of bacteria are killed more rapidly by low concentrations of penicillin than by higher concentrations (12). Eagle (13) has further observed that when bacteria are dying rapidly in optimal concentrations of penicillin and high concentrations are then added, the rate of death is promptly slowed to that characteristic of the high concentrations. On the other hand, if zone sensitive bacteria are first exposed to a high and then to a low (optimal) concentration of penicillin, the bacteria continue to die at the slow rate. Eagle postulated the inhibition by penicillin of two metabolic reactions, one at low concentrations, causing the accumulation of a toxic intermediate, and the other at high concentrations affecting earlier reactions and thereby reducing accumulation of the toxic substance. There would appear to be little difficulty in reconciling these observations with the formulations noted above.

Development of resistant organisms.—The development of bacterial resistance against penicillin is a serious clinical problem with the staphylococci only. Both in this country and abroad, the incidence of penicillin resistant staphylococci has increased progressively during recent years, until now over 50 per cent of strains isolated from hospital patients are penicillin resistant (14, 15, 16). In contrast to the resistance induced by repeated subcultures in the presence of penicillin *in vitro*, which is temporary and not associated with the production of penicillinase, resistant staphylococci isolated from patients are almost invariably penicillinase producers (15).

Efforts have been made to modify the production of penicillinase by staphylococci. It was shown, for example, by Voureka (17) and Winner (18) that when penicillinase producers were grown in the presence of penicillin sensitive organisms, resistance was abolished, and these staphylococci no longer produced penicillinase. George & Pondalai (19) later reproduced the phenomenon using extracts of bacteria, and concluded that the ribonucleic acid fraction was responsible for conferring sensitivity on the resistant organisms. Other workers (20) have not succeeded in abolishing penicillinase production in this manner, however, and thus far no practical applications have been forthcoming.

An approach more likely to have direct clinical usefulness is one recently described by Chandler *et al.* (21) who found that penicillinase production of a highly resistant *Staphylococcus* was abolished by 13 transfers in aureomycin. This now-sensitive organism was then made moderately resistant by repeated subcultures in increasing amounts of penicillin, but the resistance was of the type usually seen under these circumstances, i.e., it was not associated with the production of penicillinase. Using a different approach, it has recently been found in our laboratory (22) that there is a synergistic action between penicillin and aureomycin against penicillin resistant staphylococci, which is associated with an inhibition of penicillinase production.

A curious observation has recently been made by Clapper & Heatherman (23) who showed that when viridans streptococci become resistant to sulfathiazole following repeated subcultures, they acquired biochemical properties characteristic of enterococci, including reduction of 0.1 per cent methylene blue milk, and growth on 40 per cent bile agar and in 6.5 per cent NaCl broth. Viridans strains made resistant to aureomycin and penicillin were shown to undergo the same alterations (24). Finally, Feltz & Clapper (25) have demonstrated that those resistant streptococci possess antigens similar if not identical with those of enterococci, since positive precipitin reactions were obtained with extracts of the bacteria in the presence of *Streptococcus faecalis* antiserum.

Antibiotic spectrum and clinical studies.—No important developments have appeared concerning the therapeutic efficacy of penicillin. There is a growing tendency to administer penicillin by mouth (26), and Keefer (27) has pointed out that hypersensitivity reactions occur less commonly with the oral than the intramuscular route. Even with parenteral therapy, there seems to have been a definite decrease in hypersensitivity reactions in the last few years, according to Kitchen *et al.* (28). A new preparation, N,N-dibenzylethylenediamine penicillin, maintains measurable penicillin blood levels for many days following intramuscular injection, and is tasteless when given by mouth (29). Another preparation, Neo-penil (penicillin G diethylamino ethyl ester hydriodide) has a special affinity for lung tissue, and gives much higher spinal fluid concentrations when injected intramuscularly than does procaine penicillin (30).

Streptomycin

Mode of action.—An important study by Umbreit *et al.* (31) has recently appeared. This group had previously shown that streptomycin appears to inhibit the oxaloacetate-pyruvate reaction, thereby preventing the entrance of various compounds into the terminal respiration system of susceptible organisms (32). It was then shown by Stern & Ochoa (33) that a condensation occurred between acetate and oxaloacetate to yield citrate. It was no longer necessary to assume a direct pyruvate-oxaloacetate condensation, and the effect of streptomycin on the citrate-forming system was therefore studied. Briefly, Umbreit *et al.* (31) found that citrate formation from oxaloacetate

and pyruvate was not inhibited by streptomycin, indicating that the acetate-oxaloacetate condensation is not the only pathway by which pyruvate enters the terminal respiration system of *Escherichia coli*. A pyruvate reaction not forming citrate is presumably inhibited by streptomycin. The nature of this reaction is still unknown.

Umbreit & Tonhazy (34) also showed that, although enzymes within the mitochondria of animal cells are inhibited by streptomycin, the antibiotic can be administered to patients because barriers preventing its penetration exist at the cell wall and at the surface of the mitochondria. Magoffin & Spink (35) have recently shown that organisms of the genus *Brucella*, when localized within leucocytes, are protected from the lethal action of streptomycin. This undoubtedly accounts for the frequent relapses occurring in clinical brucellosis treated with this antibiotic.

Development of resistant organisms.—When resistance develops, the oxaloacetate-pyruvate reaction is lost (36). Other alterations may also occur, of course. English & McCoy (37) have recently shown that a streptomycin resistant *Staphylococcus* grew much better on a semisynthetic medium than did the sensitive parent. When biotin was added to the medium, the sensitive organisms grew as well as the resistant mutants. Using a screening method, the same workers isolated a streptomycin resistant variant from a sensitive culture. Since resistance appeared in the absence of streptomycin, this was regarded as evidence in favor of the mutational origin of resistance (37). Newcombe & McGregor (38) have made similar observations with *E. coli*.

Antibiotic spectrum and clinical studies.—Streptomycin is now of value chiefly for the treatment of tuberculosis. It has become routine to administer *p*-aminosalicylic acid, concomitantly to delay the emergence of resistant organisms, and the duration of therapy is being lengthened to a year or longer in many instances. Streptomycin is apparently as effective when injected three times weekly as when given daily, and this intermittent administration is helpful in decreasing the toxicity of prolonged therapy. One interesting observation, unpublished so far, is that lung tissue resected following prolonged streptomycin therapy has been found to contain tubercle bacilli which can be seen on smears, but which do not grow on culture media and do not infect guinea pigs.

Aureomycin, Terramycin, and Chloramphenicol

Mode of action.—Little is known so far concerning the mode of action of the broad spectrum antibiotics. The available information will be summarized briefly.

Smith *et al.* (39) tested the effect of chloramphenicol on 45 isolated enzyme systems. There was no inhibition of respiration of either resting or growing cells, utilization of carbohydrates through the phosphorylation cycle was not blocked, and protein breakdown was not influenced. However, esterase activity was blocked, suggesting that chloramphenicol might inhibit

the hydrolysis and synthesis of fatty acid esters. When mitochondria or animal cells were used, the action was incomplete, suggesting that a barrier exists at the cell wall, and at the surface of the mitochondria.

Loomis (40) showed that aureomycin specifically depresses phosphorylation without inhibiting respiration of normal mitochondria. Van Meter & Oleson (41) observed that respiration of whole rat liver homogenates was depressed by aureomycin when citrate was omitted from the basal medium, suggesting interference with some part of the Krebs cycle. More recently, these same workers (42), using respiring rat liver mitochondria, showed that phosphorylation of adenine nucleotides by orthophosphate was blocked in the presence of aureomycin.

The relationship of these observations to antibacterial action cannot of course be assessed at present. Umbreit (43) has observed that these three antibiotics, in common with streptomycin but not penicillin, inhibit adaptive enzyme formation more or less nonspecifically. He further points out that since they are effective against intracellular organisms, the action of these antibiotics against microorganisms is probably different than that observed with animal homogenates.

Development of resistant organisms.—Early laboratory and clinical studies with the newer antibiotics indicated that the development of resistant strains might not become an important clinical problem. However, now that all three antibiotics are being used widely, resistant strains are being isolated so commonly that prompt sensitivity testing is essential if effective therapy is to be given. Thus, over 90 per cent of staphylococci were reported by Finland *et al.* (44) and by Spink (15) to be inhibited by less than 5 μg. of aureomycin per ml. In a later study Finland (14) noted that a large percentage of staphylococci were now resistant to both aureomycin and terramycin. Similar observations have been made recently in our clinic, 50 and 45 per cent of staphylococci being resistant to 12.5 μg. per ml. or more of aureomycin and terramycin, respectively (45). Only 12 per cent were similarly resistant to chloramphenicol.

Prigal, Molomut, & Haber (46) noted that only 36 per cent of organisms isolated from sinorespiratory infections in 1951 were sensitive to aureomycin, as compared with 80 per cent in 1950. In their present study terramycin was the most effective antibiotic, inhibiting growth of 73 per cent of organisms, but it seems likely that as this antibiotic is used more widely the situation will change as it did with aureomycin. A similar decrease in sensitivity is also occurring in urinary tract infections, so much so that strains of *E. coli* highly resistant to all three antibiotics are commonly encountered.

In vitro studies indicate that there is considerable cross resistance among the newer antibiotics. Organisms developing resistance to aureomycin, chloramphenicol, or terramycin show in general a simultaneous increase in resistance to the other two antibiotics (47 to 50). The cross resistance occurs most commonly between aureomycin and terramycin and, in the

experience of Monnier & Schoenbach (51), was not observed with chloramphenicol with gram positive as opposed to gram negative organisms. Bacteria resistant to one or more of the broad spectrum antibiotics often showed increased sensitivity to streptomycin. Gocke & Finland (52) confirmed the lack of cross resistance between the three newer antibiotics and penicillin or streptomycin, but observed that neomycin resistant organisms tended to be resistant to streptomycin. It cannot be definitely stated as yet whether cross resistance occurs while patients are under therapy. One suggestive report has been published by Bergersen (53). The frequent isolation from patients of gram negative bacilli resistant to all of the broad spectrum antibiotics also suggests that the phenomenon occurs *in vivo* as well as *in vitro*.

Thus, the problem of resistance has become an important one with the newer antibiotics. Future studies, particularly of the relationship of cross resistance to the mode of action of these antibiotics, will be of great interest.

Antibiotic spectrum.—Whereas the activity of penicillin and streptomycin against various microorganisms is now well established, new information concerning the spectrum of aureomycin, chloramphenicol, and terramycin, continues to appear. It is only natural that much of this evidence is in the form of clinical reports, and both laboratory and clinical studies will therefore be considered. However, aspects not of primary interest to the microbiologist, such as dosage and methods of administration, will be ignored.

Typhoid fever is one of the few diseases for which one of these three antibiotics, chloramphenicol, is clearly superior to the other two (54, 55). Noting that *Salmonella typhosa* is equally susceptible to aureomycin and chloramphenicol *in vitro*, Werner, Knight & McDermott (56) studied the effect of these two antibiotics upon organisms imbedded in agar wafers inserted into the peritoneal cavity of a "suitable host." They found no differences in the susceptibility of the organisms to antibiotics penetrating from the extracellular fluid and concluded that the drug-host relationship of chloramphenicol assumes an importance in typhoid fever not revealed in other infections. Since both drugs were comparably active at high and inactive at low serum concentrations, and since much higher serum levels are obtained with chloramphenicol than with aureomycin, it would appear that better absorption from the intestinal tract might account for the better clinical response to chloramphenicol.

Smadel, Ley & Diercks (57) have shown that combined therapy with cortisone and chloramphenicol reduces the average febrile period in typhoid fever from four days to less than one day, as compared with chloramphenicol alone. Patients treated with cortisone alone became afebrile and atoxic almost as soon as those receiving combined therapy (58). These results are most interesting in view of evidence indicating that certain other infections are disseminated by the administration of cortisone (59, 60). A number of reports of relapses and of the persistence of positive stool cultures in typhoid fever patients have appeared recently. A good example is the paper by Matteucci, Schimmel & Boger (61), which stresses the suppressive rather than curative action of chloramphenicol.

In brucellosis, Knight *et al.* (62) have found terramycin as effective as aureomycin and chloramphenicol. The greater effectiveness of combining any one of these antibiotics with streptomycin will be discussed under antibiotic synergism. Peritonitis is another condition in which broad spectrum antibiotics have markedly reduced mortality and morbidity rates. A representative recent report is that of Rutenberg *et al.* (63).

In vitro, gram positive bacteria are in general more susceptible to aureomycin and terramycin than to chloramphenicol. It has been shown, however, that patients with pneumococcal pneumonia respond as well to chloramphenicol as to aureomycin or terramycin (64, 65). Again, this is undoubtedly due at least in part to the higher blood levels obtained with chloramphenicol.

Aureomycin has been found by Breese (66) and by Wannamaker *et al.* (67) to be highly effective in the treatment of hemolytic streptococcal infections. Indeed, in the latter study where careful controls were possible, aureomycin was more effective than penicillin in lowering the temperature and relieving symptoms. However, aureomycin was less effective than penicillin in the permanent eradication of the carrier state, which is presumably a reflection of its bacteriostatic rather than lethal action. Studies by the same group (68) suggest that aureomycin may be as effective as penicillin (69) in the prevention of acute rheumatic fever by early, intensive therapy of the streptococcal sore throat.

The problem of penicillin resistant staphylococci is considered elsewhere in this review. It may be noted here, however, that penicillin resistant cases of osteomyelitis have been managed successfully with aureomycin (70, 71) and chloramphenicol (72).

Because of its bactericidal action, penicillin is unquestionably the most effective antibiotic available for the treatment of subacute bacterial endocarditis caused by viridans streptococci. It is of interest, however, that Hamburger & Muething (73) found 11 strains of *S. viridans* recovered from patients to be quite sensitive to aureomycin and terramycin, and that a number of clinical cures have been reported with these antibiotics. Spies *et al.* (74) and Hughes (75) have described good results with aureomycin, and Arnold & Reeves (76) were successful in curing a penicillin resistant case with oral terramycin. On the other hand, a failure with terramycin was noted by Sayer *et al.* (77), and Kane & Finn (78) were able to bring about a definite cure in only two of eight patients treated with aureomycin, and in one of five treated with chloramphenicol. Similar results have recently been reported by Friedberg (79), who was able to cure only two of eight patients with aureomycin. The usual experience has been that therapy with one of the newer antibiotics is associated with prompt clinical improvement, return of the temperature to normal, and negative blood cultures. However, in the majority of instances relapse occurs within a few days after therapy is discontinued. The explanation for the cures occasionally obtained is not clear. However, the observation of Hobby and associates (80) that terramycin exerts a bactericidal action when the concentration of the antibiotic is

high, and the number of bacteria present is small, may well be applicable to the situation which occurs clinically. For the present, it would seem advisable to treat subacute bacterial endocarditis with the newer antibiotics only in penicillin resistant cases. In some instances it is undoubtedly advisable to administer aureomycin, chloramphenicol, or terramycin in conjunction with streptomycin or penicillin; this subject will be considered later in the discussion of combinations of antibiotics.

Meningococcic meningitis is one of the few remaining diseases for which the sulfonamides are preferred for therapy, and recent developments suggest that even in this instance they may gradually be replaced by the newer antibiotics. Striking results have recently been reported by McCrumb *et al.* (81) in 15 patients treated with chloramphenicol. A prompt recovery occurred in all cases, and residual signs were minimal. As was observed earlier by McLean (82), all strains of *Neisseria intracellularis* were highly susceptible *in vitro*, and in experimental mouse infections aureomycin, chloramphenicol, and terramycin all appeared to be as effective as a combination of penicillin and sulfadiazine. Terramycin has been found by Hoyne (83, 84) to produce equally favorable results, and this experienced observer feels that terramycin may be the ideal drug for combatting meningococcal infections. These results are particularly striking in view of the evidence indicating that terramycin, unlike aureomycin (85) and chloramphenicol (86), does not readily traverse the blood-brain barrier (87). Chloramphenicol (88, 89) and aureomycin (90) have also been found to be highly effective in the therapy of *Hemophilus influenzae* meningitis. It is particularly desirable in children to be able to administer a single antibiotic which is effective against both *H. influenzae* and *N. intracellularis*, since the bacteriological differentiation between these two causes of meningitis is often difficult.

Important observations have been made in relation to antibiotic therapy of amebiasis. It has been postulated that the newer antibiotics exert their action primarily on the flora of the intestine, rendering conditions unfavorable for growth of the amebae. This point of view was strengthened by the observation of Fuller & Faust (91) that strains of *Endamoeba histolytica* grew as well in cultures containing aureomycin as in penicillin-streptomycin cultures. However, it has been shown by Shaffer & Biegeleisen (92, 93), using a medium which allows the growth of *E. histolytica* without demonstrable bacterial growth, that both aureomycin and chloramphenicol have a true amebacidal action. Clinically Most & van Assendelft (94) have reported favorable results with terramycin in intestinal amebiasis with only one parasitic relapse in 37 patients. In a similar group of asymptomatic or mildly symptomatic cases, aureomycin was effective in eliminating amebic infections in 31 of 32 patients (95). In a later study by Tobie *et al.* (96), terramycin appeared to be considerably more effective than aureomycin in permanently eradicating the infection. However, because of the nature of the study, the authors felt that definite conclusions concerning this point could not be drawn. Other favorable reports concerning terramycin in

amebiasis are those of Ruiz-Sanchez and associates (97) and Knight (98). In another recent study Hoekenga (99), using only 0.75 gm. of aureomycin daily in patients with intestinal amebiasis, obtained 90 per cent apparent cures as compared with 52 per cent with carbarsone. In a group of Malayan patients treated by Ley *et al.* (100), the early response to aureomycin was good, but amebae reappeared in the stools within one month in three of eight patients. In this population it is possible that these were reinfections rather than relapses. It would appear that the broad spectrum antibiotics are highly effective in the therapy of intestinal amebiasis and may largely replace the older forms of treatment. In the presence of hepatic involvement, on the other hand, Hall (101) was able to cure only one of four patients with aureomycin. McVay *et al.* (102) and Hughes (103) had earlier reported cures in a few cases, although Hughes observed a patient with an amebic liver abscess which responded to emetine and not to aureomycin. Chloroquine or emetine should probably be used in addition to one of the newer antibiotics if the liver is involved.

Enterobiasis has recently been treated successfully with terramycin by Loughlin and associates (104), with no evidence of relapse during a five-week follow-up period in 30 patients. Ley *et al.* (100) found, on the other hand, that whipworm and hookworm infections were not affected by either aureomycin or chloramphenicol.

In experimental malaria, Darrow, Gingrich & Prine (105) observed both suppressive and protective effects when aureomycin and terramycin were administered to birds and mice. They expressed the opinion that the objective of developing a nontoxic, curative agent for relapsing vivax infections probably would not be met by any of the antibiotics currently available. However, Ruiz-Sanchez *et al.* (106) obtained favorable clinical responses in 12 *Plasmodium vivax* and 3 *P. falciparum* infections with terramycin, with no relapses in three months. Favorable clinical results have also been recently observed with aureomycin and chloramphenicol by El-Din (107).

Reports are beginning to appear of the treatment of actinomycosis with the newer antibiotics. A striking response to terramycin was recently noted by Kohn *et al.* (108) in a patient with a blood stream infection caused by an aerobic actinomycete. In this instance the infecting organism appeared to be more sensitive to terramycin than to aureomycin. McVay and associates (109) have reported a case of cervicofacial actinomycosis cured with aureomycin, and Littman *et al.* (110) cured a patient with pulmonary actinomycosis with chloramphenicol.

Terramycin administered orally has been quite effective in the therapy of acute gonorrhea. Representative reports are those of Wright *et al.* (111) in this country, who obtained a primary cure rate of 92.6 per cent, and Willcox (112) in England. Similarly favorable results have been reported with aureomycin (113, 114, 115) and chloramphenicol (116, 117). No superiority can be claimed for any one of these antibiotics at present, and it seems unlikely that they will supplant penicillin. The chief advantage of the

newer antibiotics is that they are administered orally. However, when penicillin is given by mouth in doses comparable to the newer antibiotics on the basis of weight and expense, the results are as good as those obtained with parenteral penicillin.

All three of the broad spectrum antibiotics have given excellent early therapeutic results in syphilis. In comparative studies, Robinson & Robinson (118) found that primary or secondary lesions became darkfield negative within 48 hr. with all three antibiotics, and that followup studies at six months appeared favorable. Other recent reports have also emphasized the favorable early results obtained with terramycin (119), chloramphenicol (120), and aureomycin (121). In the latter report, patients with neurosyphilis have been followed for more than a year, and Kierland & O'Leary (121) are impressed with the fact that the early benefits of aureomycin therapy are extended and maintained, and that the results thus far are equivalent to those attained with penicillin. It is obviously much too early for definitive comparisons, and further studies will be watched with interest. *Donovania granulomatis* is quite sensitive to aureomycin and chloramphenicol *in vitro* (122), and uniformly good clinical results have been reported in granuloma inguinale with aureomycin (123), terramycin (124), and chloramphenicol (125). Of particular interest, however, is the later report by Robinson & Cronk (126) who discovered a high relapse rate when patients were followed for almost two years after treatment. One patient relapsed after 531 days. They conclude that at least a year should elapse following treatment before the possibility of permanent healing can be entertained. It was their opinion that chloramphenicol gave more rapid healing of early lesions and may eventually yield a higher cure rate than aureomycin.

The beneficial effects of aureomycin in hamsters infected with *Leptospira icterohaemorrhagiae* and *Leptospira canicola*, reported by Heilman (127) and Brunner & Meyer (128), led to the hope that this antibiotic would be of value in the treatment of human leptospirosis. In isolated case reports, Brainerd *et al.* (129) and Batchelor & Todd (130) described apparently dramatic responses, although the rapid improvement was not definitely attributed to aureomycin in either instance. In another case, dramatic clinical improvement was observed within 24 hr. with terramycin therapy (131). In a thorough study of the subject made in Puerto Rico, Hall *et al.* (132) have recently reported discouraging results. Patients were treated with aureomycin, chloramphenicol, terramycin, penicillin, and streptomycin, with no demonstrable effect upon the clinical course of the disease even when therapy was begun early. It was their opinion that none of these antibiotics is worthy of future use in the treatment of patients with Weil's disease.

Loughlin, Joseph & Schaeffer (133, 134) studied the action of aureomycin and terramycin in yaws in Haiti and have reported excellent clinical results with both primary and secondary lesions. Even some tertiary lesions, which had not progressed beyond the stage of reversibility, appeared to improve

considerably. These same workers have developed a technique for studying *Treponema pertenue* by phase contrast microscopy (135) and have observed progressive nodulation and fragmentation of spirochetes removed from lesions of patients during aureomycin therapy (136). Good clinical results have also been reported with chloramphenicol (137).

A significant percentage of patients receiving the newer antibiotics develop inflammatory lesions of the vagina, vulva, anal region, and buccal mucosa. These lesions were at first considered to be avitaminoses caused by suppression of the bacterial flora of the intestinal tract (138). Harris (139), Mangiaracins (140), and Woods, Manning & Patterson (141) later isolated *Candida albicans* from a number of cases and suggested that in some instances, at least, monilia infections were being observed. An important contribution to this subject has recently been made by Pappenfort & Schnall (142), who recovered *C. albicans* from 16 successive patients who developed moniliasis-like lesions or symptoms during therapy with aureomycin. They found that aureomycin *in vitro* caused a definite stimulation of growth of *C. albicans* and that this occurred only with the oral, and not the parenteral form of the antibiotic. The growth stimulating factor seemed to be different from the antibiotic factor. It seems quite possible that this growth promoting substance is at least partially responsible for the occurrence of monilia infections during antibiotic therapy.

Conflicting results have been reported in pertussis. Rapid improvement was noted in 150 patients by Hazen *et al.* (143), with a definite reduction in the frequency, severity, and duration of paroxysmal cough. *Hemophilus pertussis* was eliminated from nasopharyngeal cultures in three to four days by the broad spectrum antibiotics, whereas with penicillin these organisms persisted for two weeks or longer. No definite superiority could be claimed for either aureomycin, chloramphenicol, or terramycin. Similar results were reported by Booher, Farrell & West (144). On the other hand, Weinstein *et al.* (145) found that the duration of illness and incidence of whooping were not notably affected by antibiotics, and that the incidence of secondary bacterial infections was not reduced appreciably by either aureomycin or chloramphenicol. Terramycin appeared to be the most effective agent in decreasing the risk of complications. Wehrle & Lepper (146) also noted little effect on the severity or duration of the paroxysmal stage, but reported a marked reduction in the incidence of secondary complications with antibiotic therapy. It is obvious that further studies utilizing careful controls are needed to clarify this situation.

The present status of the newer antibiotics in the therapy of viral and rickettsial diseases has recently been reviewed by Smadel (147). In his opinion, treatment of the rickettsial diseases has been virtually solved. The clinical results are highly satisfactory, and the only improvement which might be contemplated would be the discovery of a truly rickettsiocidal drug. The present agents are rickettsiostatic, and treatment must be continued until immunity develops. Thus, when patients with scrub typhus

were treated early in the disease for only 24 hr. with chloramphenicol, clinical manifestations were suppressed for one week, and then a recrudescence occurred. However, patients similarly treated during the second week of illness had developed sufficient immunity to prevent relapses. Smadel prefers chloramphenicol, aureomycin, and terramycin in the order listed, but notes that Ruiz-Sanchez (148) has obtained better results in the rickettsial diseases with aureomycin than with chloramphenicol. Terramycin, which has been available for a much shorter time than aureomycin or chloramphenicol, has recently been reported to be effective in the treatment of Rocky Mountain spotted fever (149), epidemic typhus (150), murine typhus (151), rickettsial pox (152), and scrub typhus (153). In experimental infections in embryonated eggs, terramycin was found by Jackson (154) and by Smadel *et al.* (155) to be much more effective than the other two agents, especially against *Coxsackie burnetti.* This is of special interest in view of the fact that Clark *et al.* (156) observed a relatively poor response to aureomycin in many patients with Q fever. In a preliminary report from Italy, Giunchi (157) has described excellent results in 10 patients with Q fever treated with terramycin, and it will be interesting to see if subsequent clinical studies bear out the laboratory evidence of the superiority of terramycin for this disease. Ormsbee (158) has compared the efficacy of rickettsiostatic agents in Q fever infections in chick embryos by the complement fixation test. This method appears to be a more sensitive indicator of antibiotic activity than death of the embryo.

The only virus infections in which the value of the newer antibiotics has been unequivocally established are psittacosis, lymphogranuloma venereum, and trachoma. Not many cases of psittacosis have been reported, but in those that have the response has been excellent (159, 160). Acute trachoma and inclusion blenorrhea respond well to topical applications of terramycin and aureomycin, but not to chloramphenicol (161, 162). Mitsui & Tanaka (161) feel that the efficacy of terramycin is considerably greater than that of aureomycin. These are interesting studies in that the two eyes were treated with different antibiotics, so that each patient served as his own control. In lymphogranuloma venereum the acute inflammatory process subsides promptly with aureomycin (163) and terramycin (164), but the results are less satisfactory in chronic cases (165). Schamberg *et al.* (163) found the results with 10 days of aureomycin therapy no better than prolonged medication with sulfonamides and suggested extending the duration of treatment.

There are two diseases, presumably viral in origin, in which early optimism concerning results of therapy with the newer antibiotics appears to have been completely unfounded. The first of these is herpes zoster. Following the early favorable report of Finland *et al.* (166), with aureomycin many observers have described what were apparently excellent responses to both aureomycin (167, 168, 169) and chloramphenicol (170, 171, 172). However, Hewitt & Williams (173) did not feel that the course of the

disease was altered in four patients by therapy with chloramphenicol, and the appearance of herpes zoster in patients receiving aureomycin for other infections was described by Schoenbach (174) and McCracken (175). The situation has recently been re-evaluated in two studies (176, 177) in which the clinical course of patients treated with aureomycin or chloramphenicol was compared with that of patients receiving placebos. There was no evidence that the antibiotics altered the course favorably in either report. Most of the patients in the control group recovered quite promptly, and the incidence of post-herpetic neuralgia was much lower than expected. The erroneous conclusions of the earlier studies apparently resulted from inadequate knowledge of the natural course of the untreated disease. A similar error was presumably responsible for the impression that patients with infectious mononucleosis responded favorably to the newer antibiotics (178, 179, 180). Here, too, control studies are now available showing that apparently dramatic responses represent the usual variability in the course of the disease, and that many patients who run a benign course can also be found in the untreated group (181, 182, 183).

Conflicting results are also continuing to appear concerning primary atypical pneumonia. Despite the favorable reports of Meiklejohn & Shragg (184) and of Collins *et al.* (185), Peck & Berry (186) observed no beneficial effects with aureomycin or chloramphenicol in a series of 141 relatively mild cases. Graves & Ball (187), however, obtained good to excellent results with each of the broad spectrum antibiotics in children who were moderately or severely ill. Difficulties in diagnosis undoubtedly account for some of the differences. Although the problem is still unsettled, most clinicians are inclined to administer antibiotics, particularly in severe cases.

Antibiotic Synergism and Antagonism

The effects of combinations of antibiotics upon microorganisms are of interest to the microbiologist from the standpoint of mode of action and to the clinician from the standpoint of therapeutic efficacy. Although this field is new and largely unexplored and is hampered by the inadequacy of the technical methods available, a few clearcut examples of synergism and antagonism have been uncovered.

Antibiotic synergism.—Perhaps the best example of antibiotic synergism is that occurring with penicillin and streptomycin against the enterococcus, first described by Robbins (188), and by Robbins & Tompsett (189). Using inhibition of growth as the endpoint, they observed "complete positive summation," or synergism, when various concentrations of the two antibiotics were combined *in vitro*. It was later shown by Jawetz (190) and Gunnison, Jawetz & Coleman (191) that penicillin and streptomycin increased the rate of killing of enterococci, as compared with penicillin alone, and that the cultures were usually sterilized by the combination. These observations were confirmed by Hunter (192). In both instances, the rate of killing was determined by performing plate counts at various

intervals. Burnell & Kirby (193), using a modified turbidimetric method which is somewhat easier to perform than plate counts, also confirmed Jawetz's findings. Direct clinical application of this phenomenon has been found in the therapy of subacute bacterial endocarditis, which in 5 to 10 per cent of instances is caused by the enterococcus. Treatment with penicillin alone (194) or streptomycin alone (195) has been disappointing, but with the combination cures have been reported in a number of cases by Hunter (196), Robbins & Tompsett (197), and Cates, Christie & Garrod (198).

The nature of the synergism of penicillin and streptomycin against the enterococcus is not known, but it may be explainable on the basis of Spicer's (199) observation that enterococci remaining viable after exposure to penicillin are susceptible to the action of streptomycin. Thus, the bacteria which normally would survive exposure to penicillin are eliminated by the concomitant administration of streptomycin. However, Jawetz was unable to show that the persisters were more susceptible to streptomycin (191). In a later study Miles *et al.* (200) observed that synergism was present only when the two antibiotics were present simultaneously and not when they acted in sequence. This suggested that synergism is not the result of modification in the bacterial population induced by one drug, making the organisms more susceptible to the other. Synergistic effects of penicillin-streptomycin mixtures have also been observed with staphylococci and group A streptococci (201) but are less striking than with enterococci (190).

Another example of synergism occurs with streptomycin and the broad spectrum antibiotics against *Brucella* organisms. Heilman (202) noted a marked reduction in the number of viable organisms in the spleens of infected mice treated with aureomycin and dihydrostreptomycin. Similar results were reported by Knight (203), who found that aureomycin gave somewhat better results when combined with streptomycin than did chloramphenicol or terramycin. Application of these animal experiments to clinical brucellosis was described by Herrell & Barber (204), whose results with aureomycin and dihydrostreptomycin were more favorable than had been achieved with any treatment used previously. Of 35 patients with culturally proved disease, 34 recovered completely after one course of therapy. In contrast, Spink, Hall & Magoffin (205) have recently reported a fairly high percentage of relapses in patients treated with streptomycin and sulfadiazine, and with aureomycin or chloramphenicol alone.

Synergism has also been observed between bacitracin and penicillin against *Staphylococcus*. Johnson & Meleney (206) observed rapid, progressive killing of organisms by concentrations of penicillin and bacitracin which did not separately, prevent multiplication. Excellent clinical results were obtained with the combination in patients who did not respond to penicillin alone (206, 207). Favorable clinical results have also been reported in the treatment of endocarditis (208, 209) and of staphylococcic meningitis (210).

Thus, three clearcut examples of a synergistic action between antibiotics have been found. In all three, a bactericidal action occurs with the

combination, and in all the phenomenon has been found to be of importance in clinical therapy.

Antibiotic antagonism.—In a series of carefully performed experiments, using enterococci, group A streptococci, and Friedlander bacilli as the test organisms, Jawetz and his associates have shown that the newer antibiotics interfere with penicillin both *in vitro* and *in vivo*. It was first observed *in vitro* that the bactericidal action of penicillin was slowed by the addition of chloramphenicol (211), and similar observations were subsequently made with aureomycin and terramycin (212). Time and dosage relationships were later described (213). The interference was always with penicillin; there was no evidence that penicillin interfered with any of the newer antibiotics. Chloramphenicol was antagonistic only in concentrations which exerted some bacteriostatic effect, and only if it reached the microorganisms prior to, or simultaneously with, penicillin. The greatest antagonistic effect was observed early, when the bactericidal action of penicillin was maximal. Later, the antagonism diminished as the killing action of penicillin decreased. Indeed, despite the interference, there was a progressive bactericidal action with the combinations which often caused ultimate sterilization of the culture.

The failure of other workers to confirm these *in vitro* observations is readily explainable on the basis of differences in the technical methods employed. Thus, Romansky *et al.* (214), instead of finding antagonism, actually observed synergism between penicillin and aureomycin against enterococci in 59 per cent of instances, and between penicillin and chloramphenicol in 19 per cent. However, their endpoint was inhibition of gross turbidity rather than rate of killing, and it may be noted that Jawetz and associates (191) obtained similar results when this method was employed. Spicer (215) who recorded inhibition of growth of organisms streaked on agar plates containing antibiotics, observed synergistic, additive, interfering, or indifferent results, depending on the combination used.

The underlying cause of the interference of antibiotics such as chloramphenicol with the bactericidal action of penicillin remains obscure. It seems quite clear that the drugs do not interact chemically or physically to neutralize each other (213). A more reasonable hypothesis is that chloramphenicol interferes with rapid microbial division and hence with optimal penicillin effect (213) since penicillin is said to exert its maximal bactericidal action on rapidly dividing organisms. It should be noted, however, that this widely accepted view concerning the action of penicillin is actually an oversimplification, since cell division is inhibited almost immediately by optimal concentrations of penicillin (216). For the moment, then, the manner in which the lethal effects of penicillin are modified by other antibiotics is little understood.

Jawetz and associates next transferred their *in vitro* observations to animal studies, infecting mice with group A streptococci since enterococci are essentially nonpathogenic for small laboratory animals (217). They

found that when penicillin was injected subcutaneously and chloramphenicol intraperitoneally, the animals receiving both drugs had a much higher mortality rate than those treated with penicillin alone. The *in vitro* interference with the early bactericidal action of penicillin was thus confirmed *in vivo*. In other mouse experiments, interference occurred when the infecting organism was *Klebsiella pneumoniae* (213) and when the interfering antibiotic was aureomycin or terramycin (218). Interference by these two antibiotics occurred in a much narrower range of concentrations than that observed with chloramphenicol, and was demonstrated only with difficulty in multiple dose schedules over a 10 hr. period (218).

In Jawetz's animal studies, effective blood levels of the antibiotics were present for only a few hours. Death or survival of the animals depended therefore on brief antibiotic action, and the results reflected the interference of chloramphenicol with the early bactericidal action of penicillin. The somewhat slower, but progressive, killing action of the combinations, which often led to complete sterilization of the cultures *in vitro* (191), played little if any role because of the short duration of antibiotic blood levels. Ahern, Burnell & Kirby (219) therefore carried out similar studies, injecting chloramphenicol and a long-acting form of penicillin repeatedly over a three-day period. Under these circumstances, interference of chloramphenicol with penicillin was not demonstrable; i.e., the mortality rate with the combination was never higher than with penicillin alone. These results were interpreted as being due to the continued bactericidal action of the combinations and, in addition, to the fact that time was allowed for effective phagocytosis to occur.

In evaluating the relationship of these observations to clinical therapy, it may be noted that in patients effective antibiotic levels are maintained continuously for many days, and that phagocytosis and other defense mechanisms play an important role in bringing about recovery. The role of the host's defenses is well illustrated by the fact that results of therapy with aureomycin, terramycin, and chloramphenicol, which are primarily bacteriostatic agents, compare favorably with those obtained with penicillin. In pneumococcal pneumonia, aureomycin has actually been reported to be superior to penicillin (220, 221). It should further be noted that the interference has always been unilateral, affecting only the action of penicillin; clinical results should therefore be at least as good as those obtained by the use of aureomycin, terramycin, or chloramphenicol alone. On the basis of this evidence, it seems reasonable to assume that administration of a broad spectrum antibiotic with penicillin should impair recovery only when the outcome depends upon the early killing action of penicillin.

One such clinical example has recently been described. Lepper & Dowling (222) reported 11 deaths among 14 patients with pneumococcal meningitis treated with penicillin and aureomycin, whereas in a control group treated with penicillin alone, only 3 of 14 patients died. Pneumococcal meningitis is

a disease which, untreated, is associated with a high mortality rate, and in which the early active killing action of penicillin has brought about more favorable results than therapy with bacteriostatic agents such as the sulfonamides and aureomycin. An explanation for the necessity of early active killing of pneumococci in the subarachnoid space may be found in the observations of Smith *et al.* (223) and Wood *et al.* (224) that surface phagocytosis (i.e., that occurring prior to opsonization by specific antibody) is dependent upon opportunities for phagocytes to entrap organisms against stationary surfaces. Such opportunities are, in general, lacking in the spinal canal. Lepper & Dowling postulated that penicillin and aureomycin are mutually antagonistic; however, interference of penicillin with aureomycin has not been described in any of the studies so far reported.

In contrast to this example of apparent interference, reports of favorable results from the administration of penicillin with a broad spectrum antibiotic have appeared. Particularly impressive to the reviewer was a patient with subacute bacterial endocarditis who had relapsed repeatedly over a period of three years despite intensive antibiotic therapy (225). Sensitivity tests indicated a synergistic action between penicillin and chloramphenicol, and a cure resulted when these two antibiotics were administered for 24 days. Altemeier & Culbertson (226) have also described instances in which penicillin and chloramphenicol appeared to provide more effective treatment.

Jawetz has also observed that combinations of antibiotics which are ordinarily antagonistic may, on occasion, have a synergistic action. Thus, although broad spectrum antibiotics were observed to manifest the same type of interference with streptomycin as with penicillin both *in vitro* and *in vivo* (227), a patient with bacterial endocarditis caused by *Staphylococcus albus* was encountered in which the infecting organisms were rapidly destroyed by terramycin and streptomycin, and a cure resulted when this combination of antibiotics was administered (228).

Further study of many bacteria has led Jawetz to certain generalizations about antibiotic antagonism and synergism (229). He has found that a given drug pair may be synergistic against one organism, and antagonistic when acting on another. Antibiotics were divided into two groups, group one consisting of penicillin, streptomycin, bacitracin, and neomycin, and group two consisting of aureomycin, chloramphenicol, and terramycin. Drugs of group one were often synergistic with each other, occasionally indifferent, never antagonistic. Drugs of group two were never synergistic with or antagonistic to each other, but frequently additive. Group two drugs frequently antagonized group one agents acting on group one susceptible organisms. Group two drugs were sometimes synergistic with group one when acting on group one resistant bacteria.

It is evident that the problem of antibiotic synergism and antagonism is quite complex and that much remains to be learned about basic mechanisms and practical applications of these important phenomena.

LITERATURE CITED

1. Brownlee, G., *Ann. Rev. Microbiol.*, **5**, 197 (1951)
2. Umbreit, W. W., *J. Mt. Sinai Hosp. N. Y.* (In press)
3. Rowley, D., Cooper, P. D., Roberts, P. W., and Lester Smith, E., *Biochem. J.*, **46**, 157 (1950)
4. Maass, E. A., and Johnson, M. J., *J. Bact.*, **57**, 415 (1949)
5. Maass, E. A., and Johnson, M. J., *J. Bact.*, **58**, 361 (1949)
6. Gale, E. F., and Taylor, E. S., *J. Gen. Microbiol.*, **1**, 314 (1947)
7. Gale, E. F., *Bull. Johns Hopkins Hosp.*, **83**, 119 (1948)
8. Hotchkiss, R. D., *Ann. N. Y. Acad. Sci.*, **53**, 13 (1950)
9. Hotchkiss, R. D., *J. Exptl. Med.*, **91**, 351 (1950)
10. Umbreit, W. W. (Personal communication)
11. Paine, T. F., Jr., *J. Bact.*, **61**, 259 (1951)
12. Eagle, H., and Musselman, A. D., *J. Exptl. Med.*, **88**, 99 (1948)
13. Eagle, H., *J. Bact.*, **62**, 663 (1951)
14. Finland, M., *Bull. N. Y. Acad. Med.*, **27**, 199 (1951)
15. Spink, W. W., *J. Lab. Clin. Med.*, **37**, 1 (1951)
16. Barber, M., and Rozwadowska, D. M., *Lancet*, **II**, 641 (1948)
17. Voureka, A., *Lancet*, **I**, 62 (1948)
18. Winner, H. I., *Lancet*, **I**, 674 (1948)
19. George, M., and Pondalai, K. M., *Lancet*, **I**, 955 (1949)
20. Barber, M., *Lancet*, **I**, 674 (1948)
21. Chandler, C. A., Davidson, V. Z., Long, P. H., and Monnier, J. J., *Bull. Johns Hopkins Hosp.*, **89**, 81 (1951)
22. Kirby, W. M. M. (Unpublished data)
23. Clapper, W. E., and Heatherman, M. E., *Proc. Soc. Exptl. Biol. Med.*, **73**, 153 (1950)
24. Heatherman, M. E., and Clapper, W. E., *J. Lab. Clin. Med.*, **38**, 881 (1951)
25. Feltz, M., and Clapper, W. E., *Proc. Soc. Exptl. Biol. Med.*, **78**, 491 (1951)
26. Bunn, P., Caldwell, E. R., Adair, C., Lepper, M., and Dowling, H., *J. Am. Med. Assoc.*, **144**, 1540 (1950)
27. Keefer, C. S., *Postgrad. Med.*, **9**, 101 (1951)
28. Kitchen, D. K., Rein, C. R., Thomas, E. W., and Spoor, H. J., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **35**, 578 (1951)
29. Elias, W., Price, A. H., and Merrion, H. J., *Antibiotics and Chemotherapy*, **1**, 491 (1951)
30. Jensen, K. A., Dragsted, P. J., Kiaer, I., Nielsen, E. J., and Frederiksen, E., *Ugeskrift Laeger*, **113**, 1035 (1951)
31. Umbreit, W. W., Smith, P. H., and Oginsky, E. L., *J. Bact.*, **61**, 595 (1951)
32. Umbreit, W. W., *J. Biol. Chem.*, **177**, 703 (1949)
33. Stern, J. R., and Ochoa, S., *J. Biol. Chem.*, **179**, 491 (1949)
34. Umbreit, W. W., and Tonhazy, N. E., *J. Bact.*, **58**, 769 (1949)
35. Magoffin, R. L., and Spink, W. W., *J. Lab. Clin. Med.*, **37**, 2 (1951)
36. Smith, P. H., Oginsky, E. L., and Umbreit, W. W., *J. Bact.*, **58**, 761 (1949)
37. English, A. R., and McCoy, E., *J. Bact.*, **62**, 19 (1951)
38. Newcombe, H. B., and McGregor, J., *J. Bact.*, **62**, 539 (1951)
39. Smith, G. N., Worrel, C. S., and Swanson, A. L., *J. Bact.*, **58**, 803 (1949)
40. Loomis, W. F., *Science*, **111**, 474 (1950)
41. Van Meter, J. C., and Oleson, J. J., *Science*, **113**, 273 (1951)

42. Van Meter, J. C., Spector, A., Williams, J. H., and Oleson, J. J. (Unpublished data)
43. Umbreit, W. W. (Personal communication)
44. Finland, M., Frank, P. F., and Wilcox, C., *Am. J. Clin. Path.*, **20**, 325 (1950)
45. Ahern, J. J., and Kirby, W. M. M. (Unpublished data)
46. Prigal, S. J., Molomut, N., and Haber, A., *Arch. Otolaryngol.*, **54**, 493 (1951)
47. Fusillo, M. H., and Romansky, M. J., *Antibiotics and Chemotherapy*, **1**, 107 (1951)
48. Kaipainen, W. J., *Ann. Med. Exptl. Biol. Fenniae*, **29**, 15 (1951)
49. Gezon, H. M., and Fasan, D. M., *Science*, **114**, 422 (1951)
50. Pansy, F. E., Khan, P., Pagano, J. F., and Donovick, R., *Proc. Soc. Exptl. Biol. Med.*, **75**, 1618 (1950)
51. Monnier, J. J., and Schoenbach, E. B., *Antibiotics and Chemotherapy*, **1**, 472 (1951)
52. Gocke, T. M., and Finland, M., *J. Lab. Clin. Med.*, **38**, 719 (1951)
53. Bergersen, F., *Nature*, **167**, 995 (1951)
54. Knight, V., Ruiz-Sanchez, F., Ruiz-Sanchez, A., Shultz, S., and McDermott, W., *Arch. Internal Med.*, **85**, 44 (1950)
55. Smadel, J. E., Bailey, C. A., and Lewthwaite, R., *Ann. Internal Med.*, **33**, 1 (1950)
56. Werner, C. A., Knight, V., and McDermott, W., *J. Clin. Invest.*, **30**, 682 (1951)
57. Smadel, J. E., Ley, H. L., Jr., and Diercks, F. H., *Ann. Internal Med.*, **34**, 1 (1951)
58. Woodward, T. E., Hall, H. E., Dias-Rivera, R., Hightower, J. A., Martinez, E., and Parker, R. T., *Ann. Internal Med.*, **34**, 10 (1951)
59. Thomas, L., and Good, R. A., *Proc. Soc. Exptl. Biol. Med.*, **76**, 604 (1951)
60. Glaser, R. J., Berry, J. W., Loeb, L. H., and Wood, W. B., Jr., *J. Clin. Invest.*, **30**, 640 (1951)
61. Matteucci, W. V., Schimmel, N. H., and Boger, W. P., *Am. J. Med. Sci.*, **222**, 446 (1951)
62. Knight, V., Ruiz-Sanchez, F., and Ruiz-Sanchez, A., *Arch. Internal Med.*, **87**, 835 (1951)
63. Rutenberg, A., Jacob, S. W., Schweinburg, F. B., and Fine, J., *New Engl. J. Med.*, **246**, 52 (1952)
64. Flippin, H. F., Matteucci, W. V., Schimmel, N. H., and Boger, W. P., *J. Am. Med. Assoc.*, **147**, 918 (1951)
65. Kirby, W. M. M., Michel, J. C., Coleman, D. H., Haviland, J. W., and Sparkman, D. R., *J. Am. Med. Assoc.*, **147**, 110 (1951)
66. Breese, B. B., *J. Pediat.*, **40**, 85 (1952)
67. Wannamaker, L. W., Brink, W. R., Denny, F. W., and Rammelkamp, C. H., Jr., *Am. J. Med.*, **10**, 237 (1951)
68. Rammelkamp, C. H. (Personal communication)
69. Wannamaker, L. W., Rammelkamp, C. H., Jr., Denny, F. W., Brink, W. R., Houser, H. B., and Hahn, E. O., *Am. J. Med.*, **10**, 673 (1951)
70. Buchman, J., and Blair, J. E., *J. Bone Joint Surg.*, **33**, 107 (1951)
71. Wright, L. T., *J. Natl. Med. Assoc.*, **43**, 104 (1951)
72. Bernard, A., Henninot, E., and Pauli, M., *J. sci. méd. Lille*, **60**, 376 (1951)
73. Hamburger, M., and Muething, D., *J. Lab. Clin. Med.*, **37**, 60 (1951)
74. Spies, H. W., Dowling, H. F., Lepper, M. H., Wolfe, C. K., and Caldwell, E. R., Jr., *Arch. Internal Med.*, **87**, 66 (1951)

75. Hughes, S. O., *Am. J. Med.*, **10**, 402 (1951)
76. Arnold, W. P., Jr., and Reeves, R. L., *Antibiotics and Chemotherapy*, **1**, 181 (1951)
77. Sayer, R. J., Michel, J. C., Moll, F. C., and Kirby, W. M. M., *Am. J. Med. Sci.*, **221**, 256 (1951)
78. Kane, L. W., and Finn, J. J., *New Engl. J. Med.*, **244**, 623 (1951)
79. Friedberg, C. K., *J. Am. Med. Assoc.*, **148**, 98 (1952)
80. Hobby, G. L., Lenert, T. F., Pikula, D., Kiseluk, M., and Hudders, M. E., *Ann. N. Y. Acad. Sci.*, **53**, 266 (1950)
81. McCrumb, F. R., Hall, H. E., Merideth, A. M., Deane, G. E., Minor, J. V., Jr., and Woodward, T. E., *Am. J. Med.*, **10**, 696 (1951)
82. McLean, J. W., Jr., Schwab, J. L., Hillegas, A. B., and Schlingman, A. S., *J. Clin. Invest.*, **28**, 953 (1949)
83. Hoyne, A. L., and Riff, E. R., *J. Pediat.*, **39**, 151 (1951)
84. Hoyne, A. L., *Illinois Med. J.*, **100**, 285 (1951)
85. Herrell, W. E., and Heilman, F. R., *Proc. Staff Meetings Mayo Clinic*, **24**, 157 (1949)
86. Ross, S., Bischoff, H., Preisser, W., and Orr, W., *J. Clin. Invest.*, **28**, 1049 (1949)
87. Herrell, W. E., Heilman, F. R., and Wellman, W. E., *Ann. N. Y. Acad. Sci.*, **53**, 448 (1950)
88. Prather, G. W., and Smith, M. H. D., *J. Am. Med. Assoc.*, **143**, 1405 (1950)
89. McCrumb, F. R., Hall, H. E., Imburg, J., Merideth, A. M., Helmhold, R., Basora y Defillo, J., and Woodward, T. E., *J. Am. Med. Assoc.*, **145**, 469 (1951)
90. Drake, M. E., Bradley, J. E., Imburg, J., McCrumb, F. R., and Woodward, T. E., *J. Am. Med. Assoc.*, **142**, 463 (1950)
91. Fuller, F. W., and Faust, E. C., *Science*, **110**, 509 (1949)
92. Shaffer, J. G., and Biegeleisen, J. Z., *Am. J. Hygiene*, **53**, 139 (1951)
93. Biegeleisen, J. Z., and Shaffer, J. G., *Am. J. Hygiene*, **53**, 146 (1951)
94. Most, H., and Assendelft, F. van, *Am. J. Trop. Med.*, **31**, 284 (1951)
95. Most, H., Miller, J. W., and Grossman, E. J., *Am. J. Trop. Med.*, **30**, 491 (1950)
96. Tobie, J. E., Most, H., Reardon, L. V., and Bozicevich, J., *Am. J. Trop. Med.*, **31**, 414 (1951)
97. Ruiz-Sanchez, F., Riebeling, R., and Arreola, E. C., *Med. Rev. Mexicana*, **30**, 365 (1950)
98. Knight, V., *N. Y. State J. Med.*, **50**, 2173 (1950)
99. Hoekenga, M. T., *Am. J. Trop. Med.*, **31**, 420 (1951)
100. Ley, H. L., Sayer, W. J., Hobson, A. C. S., Vanreenen, R. M., Tipton, V. J., Frick, L. P., Ballard, E. L., and Traub, R., *Antibiotics and Chemotherapy*, **1**, 281 (1951)
101. Hall, W. H., *New Engl. J. Med.*, **244**, 495 (1951)
102. McVay, L. V., Laird, R. L., and Sprunt, D. H., *Science*, **109**, 590 (1949)
103. Hughes, J. D., *J. Am. Med. Assoc.*, **142**, 1052 (1950)
104. Loughlin, E. H., Rappaport, I., Mullin, W. G., Wells, H. S., Joseph, A. A., and Shookhoff, H. B., *Antibiotics and Chemotherapy*, **1**, 588 (1951)
105. Darrow, E. M., Gingrich, W. D., and Prine, J. H., *Natl. Malaria Soc. Meeting*, Chicago, Ill., Nov. 15, 1951)
106. Ruiz-Sanchez, F., Casillas, J., Paredes, E. M., Velazquez, J., and Riebeling, R., *Antibiotics and Chemotherapy*, **2**, 51 (1952)
107. El-Din, G. N., *J. Roy. Egypt. Med. Assoc.*, **34**, 444 (1951)

108. Kohn, P. M., Tager, M., Siegel, M. L., and Ashe, R., *New Engl. J. Med.*, **245**, 640 (1951)
109. McVay, L. V., Dunavont, D., Guthrie, F., and Sprunt, D. H., *J. Am. Med. Assoc.*, **143**, 1067 (1950)
110. Littman, M. W., Paul, J. S., and Fusillo, M. H., *J. Am. Med. Assoc.*, **148**, 608 (1952)
111. Wright, L. T., Prigot, A., DiLorenzo, J. C., Whitaker, J. C., and Marmell, M., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **35**, 490 (1951)
112. Willcox, R. R., *Brit. Med. J.*, **II**, 527 (1951)
113. Collins, H. S., Trousdale, H., Kaiser, T. F., Regan, F. C., and Finland, M., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **33**, 263 (1949)
114. Robinson, R. C. V., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **34**, 64 (1950)
115. Robinson, R. C. V., and Galen, W. P., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **35**, 488 (1951)
116. Smadel, J. E., Bailey, C. A., and Mankiker, D. S., *J. Clin. Invest.*, **28**, 964 (1949)
117. Robinson, H. M., and Robinson, H. M., Jr., *Southern Med. J.*, **42**, 989 (1949)
118. Robinson, H. M., and Robinson, H. M., Jr., *Arch. Dermatol. and Syphilol.*, **63**, 687 (1951)
119. Robinson, H. M., and Robinson, H. M., Jr., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **35**, 479 (1951)
120. Romansky, M. J., Olansky, S., Taggart, S. R., Landman, G. S., and Robin, E. D., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **35**, 234 (1951)
121. Kierland, R. R., and O'Leary, P. A., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **35**, 544 (1951)
122. Chen, C. H., Dienst, R. B., and Greenblatt, R. B., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **35**, 383 (1951)
123. Robinson, R. C. V., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **34**, 273 (1950)
124. Whitaker, J. C., Wright, L. T., Beinfield, M. S., Wilkinson, R. S., and Marmell, M., *Antibiotics and Chemotherapy*, **1**, 208 (1951)
125. Greenblatt, R. B., Wammock, V. S., Dienst, R. B., and West, R. M., *Am. J. Obstet. Gynecol.*, **59**, 1129 (1950)
126. Robinson, R. C. V., and Cronk, B., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **35**, 378 (1951)
127. Heilman, F. R., *Proc. Staff Meetings May Clinic*, **23**, 569 (1948)
128. Brunner, K. T., and Meyer, K. F., *Am. J. Vet. Research*, **11**, 89 (1950)
129. Brainerd, H., Lennette, E. H., Meiklejohn, G., Bruyn, H. B., and Clark, W. H., *J. Clin. Invest.*, **28**, 992 (1949)
130. Batchelor, T. M., and Todd, G. M., *J. Am. Med. Assoc.*, **143**, 21 (1950)
131. Liebowitz, D., and Schwartz, H., *J. Am. Med. Assoc.*, **147**, 122 (1951)
132. Hall, H. E., Hightower, J. A., Dia-Rivera, R., Byrne, R. J., Smadel, J. E., and Woodward, T. E., *Ann. Internal Med.*, **35**, 981 (1951)
133. Loughlin, E. H., Joseph, A., and Schaeffer, K., *Am. J. Trop. Med.*, **31**, 20 (1951)
134. Loughlin, E. H., and Joseph, A., *Antibiotics and Chemotherapy*, **1**, 76 (1951)
135. Schaeffer, K., Loughlin, E. H., and Joseph, A., *Am. J. Trop. Med.*, **31**, 26 (1951)
136. Schaeffer, K., Loughlin, E. H., and Joseph, A., *Am. J. Trop. Med.*, **31**, 24 (1951)
137. Payne, E. H., Bellerive, A., and Lafont, J., *Antibiotics and Chemotherapy*, **1**, 88 (1951)
138. O'Leary, P. A., Kierland, R. R., and Herrell, W. E., *Proc. Staff Meetings Mayo Clinic*, **23**, 574 (1948)
139. Harris, H. J., *J. Am. Med. Assoc.*, **142**, 161 (1950)

140. Mangiaracins, A., *New Engl. J. Med.*, **244,** 655 (1951)
141. Woods, J. W., Manning, I. H., and Patterson, C. N., *J. Am. Med. Assoc.*, **145,** 207 (1951)
142. Pappenfort, R. B., Jr., and Schnall, E. S., *Arch. Internal Med.*, **88,** 729 (1951)
143. Hazen, L. N., Jackson, G. G., Chang, S., Place, E. H., and Finland, M., *J. Pediat.*, **39,** 1 (1951)
144. Booher, C. E., Farrell, J. B., and West, E. J., *J. Pediat.*, **38,** 411 (1951)
145. Weinstein, L., Seltser, R., and Marrow, C. T., *J. Pediat.*, **39,** 549 (1951)
146. Wehrle, P. F., and Lepper, M. H., *J. Pediat.*, **39,** 435 (1951)
147. Smadel, J. E., *Bull. N. Y. Acad. Med.*, **27,** 221 (1951)
148. Ruiz-Sanchez, F., *Med. Rev. Mexicana*, **30,** 165 (1950)
149. Powell, A. M., Snyder, M. J., Minor, J. V., Jr., Benson, J. F., and Woodward, T. E., *Bull Johns Hopkins Hosp.*, **89,** 30 (1951)
150. Killough, J. H., and Magill, G. B., *J. Am. Med. Assoc.*, **147,** 1737 (1951)
151. Bauer, R. E., Parker, R. T., Hall, H. E., Benson, J. F., Joslin, B. S., Hightower, J. A., Snyder, M. J., Venable, S. J., and Woodward, T. E., *Ann. N. Y. Acad. Sci.*, **53,** 395 (1950)
152. Rose, H. M., *Ann. N. Y. Acad. Sci.*, **53,** 385 (1950)
153. Bailey, C. A., Ley, H. L., Jr., Diercks, F. H., Lewthwaite, R., and Smadel, J. E., *Antibiotics and Chemotherapy*, **1,** 16 (1951)
154. Jackson, E. B., *Antibiotics and Chemotherapy*, **1,** 231 (1951)
155. Smadel, J. E., Jackson, E. B., and Ley, H. L., Jr., *Ann. N. Y. Acad. Sci.*, **53,** 375 (1950)
156. Clark, W. H., Lennette, E. H., and Meiklejohn, G., *Arch. Internal Med.*, **87,** 204 (1951)
157. Giunchi, G., *Minerva Med.*, **42,** 829 (1951)
158. Ormsbee, R. A., and Pickens, E. G., *J. Immunol.*, **67,** 437 (1951)
159. Green, T. W., *J. Am. Med. Assoc.*, **144,** 237 (1950)
160. Hamke, H., *Deutsche med. Wochschr.*, **76,** 246 (1951)
161. Mitsui, Y., and Tanaka, C., *Antibiotics and Chemotherapy*, **1,** 146 (1951)
162. Mitsui, Y., Tanaka, C., Toya, H., Iwashige, Y., and Yamashita, K., *Arch. Ophthalmol. (Chicago)*, **46,** 235 (1951)
163. Schamberg, I. L., Carrozzino, O. M., and Boger, W. P., *Am. J. Syphilis, Gonorrhea, Venereal Diseases*, **35,** 370 (1951)
164. Wright, L. T., Whitaker, J. C., Wilkinson, R. S., and Beinfield, M. S., *Antibiotics and Chemotherapy*, **1,** 193 (1951)
165. Fletcher, A., Sigel, M. M., and Zintel, H. A., *Arch. Surg.*, **62,** 239 (1951)
166. Finland, M., Finnerty, E. F., Jr., Collins, H. S., Baird, J. W., Gocke, T. M., and Kass, E. H., *New Engl. J. Med.*, **241,** 1037 (1949)
167. Rosenberg, W. A., *Arch. Dermatol. and Syphilol.*, **63,** 134 (1951)
168. Benedek, T., *Military Surgeon*, **108,** 491 (1951)
169. Pritikin, R. I., and Duchon, M. L., *Am. J. Ophthalmology*, **34,** 893 (1951)
170. Babbage, N. F., *Med. J. Australia*, **I,** 339 (1951)
171. Beil, W. C., Keith, F., and Mims, J. L., Jr., *Am. J. Ophthalmology*, **34,** 623 (1951)
172. St. John, B. D., *N. Y. State J. Med.*, **50,** 1112 (1950)
173. Hewitt, W. L., and Williams, B., Jr., *New Eng. J. Med.*, **242,** 119 (1950)
174. Schoenbach, E. B., *J. Mt. Sinai Hosp. N. Y.*, **16,** 71 (1949)
175. McCracken, J. P., *New Engl. J. Med.*, **243,** 760 (1950)
176. Carter, A. B., *Brit. Med. J.*, **I,** 987 (1951)

177. Kass, E. H., Aycock, R. R., and Finland, M., *New Engl. J. Med.*, **246**, 167 (1952)
178. Gruskin, B. J., *Ann. Internal Med.*, **31**, 678 (1949)
179. Lyons, H. A., and Hard, E. M., *Bull. N. Y. Acad. Med.*, **26**, 279 (1950)
180. Carter, C. H., and Sydenstricker, V. P., *Am. J. Med.*, **10**, 309 (1951)
181. Cronk, G. A., *Am. J. Med. Sci.*, **222**, 413 (1951)
182. Burnett, J. B., and Milne, J., *New Engl. J. Med.*, **245**, 495 (1951)
183. Seifert, M. H., Chandler, V. L., and Van Winkle, W., Jr., *J. Am. Med. Assoc.*, **142**, 1133 (1950)
184. Meiklejohn, G., and Shragg, R. I., *J. Am. Med. Assoc.*, **140**, 391 (1949)
185. Collins, H. S., Wells, E. B., Gocke, T. M., and Finland, M., *Am. J. Med.*, **8**, 4 (1950)
186. Peck, G. A., and Berry, J. W., *Antibiotics and Chemotherapy*, **1**, 291 (1951)
187. Graves, F. B., and Ball, W. O., *J. Pediat.*, **39**, 151 (1951)
188. Robbins, W. C., *J. Clin. Invest.*, **28**, 806 (1949) (Abstract)
189. Robbins, W. C., and Tompsett, R., *J. Clin. Invest.* **28**, 1043 (1949)
190. Jawetz, E., *Science*, **111**, 254 (1950)
191. Gunnison, J. B., Jawetz, E., and Coleman, V. R., *J. Lab. Clin. Med.*, **36**, 900 (1950)
192. Hunter, T. H., *J. Am. Med. Assoc.*, **144**, 524 (1950)
193. Burnell, J. M., and Kirby, W. M. M., *Antibiotics and Chemotherapy*, **1**, 405 (1951)
194. Loewe, L., Candel, S., and Eibert, H. B., *Ann. Internal Med.*, **34**, 717 (1951)
195. Hunter, T. H., *Am. J. Med.*, **2**, 436 (1947)
196. Hunter, T. H., *Am. J. Med.*, **1**, 83 (1946)
197. Robbins, W. C., and Tompsett, R., *Am. J. Med.*, **10**, 278 (1951)
198. Cates, J. E., Christie, R. V., and Garrod, L. P., *Brit. Med. J.*, **I**, 653 (1951)
199. Spicer, S., and Blitz, D., *J. Lab. Clin. Med.*, **33**, 417 (1948)
200. Miles, C. P., Coleman, V. R., Gunnison, J. B., and Jawetz, E., *Proc. Soc. Exptl. Biol. Med.*, **78**, 738 (1951)
201. Nichols, A. C., *Proc. Soc. Exptl. Biol. Med.*, **69**, 477 (1948)
202. Heilman, F. R., *Proc. Staff Meetings Mayo Clinic*, **24**, 133 (1949)
203. Knight, V., *Ann. N. Y. Acad. Sci.*, **53**, 332 (1950)
204. Herrell, W. E., and Barber, T. E., *J. Am. Med. Assoc.*, **144**, 519 (1950)
205. Spink, W. W., Hall, W. H., and Magoffin, R., *Arch. Internal Med.*, **88**, 419 (1951)
206. Johnson, B. A., and Meleney, F. L., *Ann. N. Y. Acad. Sci.*, **53**, 42 (1950)
207. Meleney, F. L., Johnson, B. A., and Teng, P., *J. Mt. Sinai Hosp. N. Y.*, **18**, 257 (1952)
208. Volini, I. F., and Kadison, E. R., *Am. Practitioner*, **2**, 13 (1951)
209. Friedberg, C. K., and Bader, M. E., *J. Am. Med. Assoc.*, **147**, 46 (1951)
210. Teng, P., and Meleney, F. L., *Surgery*, **28**, 516 (1950)
211. Jawetz, E., Gunnison, J. B., and Coleman, V. R., *Science*, **111**, 254 (1950)
212. Gunnison, J. B., Coleman, V. R., and Jawetz, E., *Proc. Soc. Exptl. Biol. Med.*, **75**, 183 (1950)
213. Jawetz, E., Gunnison, J. B., Speck, R. S., and Coleman, V. R., *Arch. Internal Med.*, **87**, 349 (1951)
214. Romansky, M. J., Fusillo, B. S., Caldwell, R., and Robin, E. D., *Med. Clinics N. Amer.*, **35**, 535 (1951)
215. Spicer, S., *J. Lab. Clin. Med.*, **36**, 183 (1950)
216. Eagle, H., and Musselman, A. D., *J. Bact.*, **58**, 475 (1949)

217. Jawetz, E., and Speck, R. S., *Proc. Soc. Exptl. Biol. Med.*, **74**, 93 (1950)
218. Speck, R. S., Jawetz, E., and Gunnison, J. B., *Arch. Internal Med.*, **88**, 168 (1951)
219. Ahern, J. J., Burnell, J. M., and Kirby, W. M. M., *Proc. Soc. Exptl. Biol. Med.* (In press)
220. Gocke, T. M., Collins, H. S., and Finland, M., *Arch. Internal Med.*, **84**, 857 (1949)
221. Dowling, H. F., Lepper, M. H., Hussey, H. H., Caldwell, E. R., and Spies, H. W., *J. Lab. Clin. Med.*, **35**, 215 (1950)
222. Lepper, M. H., and Dowling, H. F., *Arch. Internal Med.*, **88**, 489 (1951)
223. Smith, M. R., Perry, W. D., Berry, J. W., and Wood, W. B., Jr., *J. Immunol.*, **67**, 71 (1951)
224. Wood, W. B., Jr., Smith, M. R., Perry, W. D., and Berry, J. W., *J. Exptl. Med.*, **94**, 521 (1951)
225. Ahern, J. J., and Kirby, W. M. M. (Unpublished data)
226. Altemeier, W. A., and Culbertson, W. R., *J. Am. Med. Assoc.*, **145**, 449 (1951)
227. Jawetz, E., Gunnison, J. B., and Speck, R. S., *Am. J. Med. Sci.*, **222**, 404 (1951)
228. Jawetz, E., Gunnison, J. B., and Speck, R. S., *New Engl. J. Med.*, **245**, 966 (1951)
229. Jawetz, E., and Gunnison, J. B., *Abstracts Western Soc. Clin. Research* (Carmel, Calif., Jan. 26, 1952)

THE MODE OF ACTION OF CHEMOTHERAPEUTIC AGENTS[1]

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Chemotherapy, i.e., the therapy of infectious diseases by means of chemically definable substances, is very old. Understanding of the problems involved in this field has a much shorter history. Let us assume the beginning of our understanding to be due to Ehrlich, who gave us the magnificent but dangerous explanatory hypothesis of the sidechain theory. Magnificent, because it opened the way of thinking in the matter; dangerous, because it gave an explanation of the phenomena and their laws. The theory was a "passe-partout"; explaining everything is, after all, explaining nothing. Thus, magnificent though it has been, it has delayed progress for a considerable time. This situation did not change until after the introduction of the sulfonamides in chemotherapy, especially so when the action of these drugs proved to be demonstrable *in vitro*. The rapid multiplication of bacteria and the relative ease with which they may be studied *in vitro* as compared to protozoa and viruses, offered a hitherto unknown possibility of studying the mode of action of chemotherapeutic agents. From that moment, great progress has been made.

Search for chemotherapeutic drugs, on the other hand, is still purely empirical. Denudated of all imponderable thoughts and suggestive ideas of any particular investigator, research still is forced to follow the classical scheme. The infection under consideration has to be induced in experimental animals; the drug in question has to be administered to the diseased animals; and we have to see whether they will respond favorably. Then may follow tests to determine whether the substance may be of any value in human beings. But this last step is not essential: to include any drug among the chemotherapeutics, it is sufficient to establish its therapeutic potency in an infected animal. In the past this manner of evaluation was the only one used. Since the revolution in the methods of studying chemotherapeutics, mentioned above, a preliminary test has been introduced based on the fact that the infecting agent is influenced by the drugs *in vitro*. At the present time much stress is laid on the killing of microorganisms or hampering of their multiplication *in vitro;* practically every search for new chemotherapeutics is initiated by such screening. However, it must be kept in mind that any antiseptic, disinfectant, or the like, will kill or inhibit microorganisms. We must admit that even now we do not know what the difference may be between an antiseptic or disinfectant on the one hand and a chemotherapeutic on the other hand. If we knew the answer to this fundamental question, chemotherapy doubtless

[1] The survey of the literature pertaining to this review was concluded in October, 1951.

would be entirely different from what it is today. The preliminary *in vitro* test thus has to be followed by the *in vivo* experiment. Among the innumerable antibacterial substances, whether they be of biological or of purely synthetic origin, there are relatively few of any chemotherapeutic value, and as a matter of fact very few that are of real value. This is not surprising for we do not understand why a substance is a chemotherapeutic. The only thing we dare say is that the point of attack of chemotherapeutics against infectious agents must be "specific"; i.e., the microorganism is dependent on that very part of its utterly complicated metabolic equipment that is suppressed by the substance with chemotherapeutic properties, while for the host this suppression is of little or no significance.

When a substance is found that shows chemotherapeutic activity, even if it is feeble and unsatisfactory, all possible attention is paid to its mode of action. This generally will be done *in vitro* as it is difficult to study it when we have to take into account the complicated environment of the diseased host. But this approach carries its misleading factors. When we find that a chemotherapeutic produces a disturbance in the physiological behaviour of microorganisms, this may or may not be the essential feature that endows the substance with its special properties. So we will feel many restrictions in considering this *in vitro* effect as the clue to the mode of action of the drug. As an example, let us take suppression of bacterial respiration. Many agents have this effect, including too high or too low a temperature, inadequate pH, antiseptics, or the lack of a vital constituent in the medium in which the experiment is performed. Generally speaking, the most valuable experiments are the ones in which all circumstances are as close as possible to those in the host. But even then it has to be proved that the phenomena observed are specific or characteristic. In other words it has to be checked whether other antibacterial agents, valueless as chemotherapeutics, will or will not have the same or comparable effects on the microorganisms. Frequently this fundamental control is lacking. A synthesis of the different data may then give us some concept of chemotherapy; of course we are far from that point. The future will have to decide as to the real meaning of the imposing and overwhelming number of facts this most fruitful field has disclosed to us. One thing may be accepted as certain: of the many facts that are coming to our knowledge some will and some will not be clues to the problem of chemotherapy, but all are of importance to our knowledge of microbial physiology and biochemistry. Chemotherapy thus has enriched the field of microbiology to an unforeseen extent, somewhat as pharmacology has given support to physiology. The risk of the reviewer is that facts of no importance to chemotherapy may be mentioned and others seemingly of no relationship to our subject, but in fact of essential importance, may be overlooked.

For the time being we can do no better than assume that influences on bacteria of substances endowed with chemotherapeutic potency will have something to do with the mode of action of chemotherapeutics, knowing that in fact we are groping in the dark.

An attempt has been made to classify the influence of chemotherapeutics on microorganisms into functions and phenomena. The question whether or not the points under consideration are essential to chemotherapy will have to be left open.

The following headings can be drawn from the literature on the subject:

I. Morphological changes
II. Multiplication and its suppression
III. Microbial respiration
IV. Metabolism (other than respiration)
  a. general
  b. specific metabolic interference
    (1) protein metabolism (including amino acids)
    (2) growth factors
    (3) special enzyme systems
V. Resistance to chemotherapeutics
VI. Physical and physicochemical factors
VII. Interrelationship of chemotherapeutic drugs, synergism, antagonism
VIII. Chemotherapeutics, parasite and host
IX. Miscellanea

Many reviews have covered the field previously and references to earlier literature may be found therein (1 to 4).

Morphological Changes

Abnormal bacterial forms caused by penicillin have been described many times. The most comprehensive studies were made by Fleming and co-workers (5). *Proteus vulgaris* showed most fantastic morphological changes when cultured in the presence of penicillin. Photomicrographs illustrate the findings in a fascinating way.

Hunter, Mudd & Woodburn (6) studied three sulfathiazole-resistant strains of staphylococci. The individual cells were more pleomorphic and generally larger than those of the parent strains. Their nuclei were somewhat more readily stainable after hydrolysis with hydrochloric acid. The nuclear structure was essentially unchanged.

Crofts & Evans (7) and Johnstone, Crofts & Evans (8) studied the influence of penicillin on the morphology of *Clostridium welchii* type A (*C. perfringens*) and observed elongation of the cells which later lost their gram positive character. Continued action induced lysis and death. The cells isolated in their abnormal forms returned to normal when transferred and subcultured in penicillin-free medium. Their biological properties as far as studied were not changed as compared to the original strains.

Benda & Urquia (9), studying tubercle bacilli by a special staining method in sputa of patients treated with streptomycin, saw a difference in morphology and staining reaction of the resistant bacilli as compared with

the original ones. Linz (10) confirmed this, but observed that the difference disappeared in cultures of the resistant organisms. Reinjected in guinea pigs the difference was clearly established again.

The morphology of *Candida albicans* changed under the influence of aureomycin [Moore (11)]. Giant forms developed in high concentrations; small and rapidly mutliplying forms developed in diluted aureomycin.

Ormerod (12) working with trypanosomes saw basophilic inclusion bodies in their cytoplasm when antrycide in therapeutic dose was administered to infected animals. Phase contrast microscopy demonstrated the bodies in the living parasites. He concluded that the action of the drug is not primarily on the nucleus.

Modern developments in microbiology have to some extent led away from direct morphological observation. Some interesting features may however be detected although their interpretation will not be at hand.

Phenomena in Multiplication and its Suppression

One of the essential features in the action of chemotherapeutics on microorganisms is suppression of growth. In an earlier period chemotherapeutics were compared to disinfectants. These latter substances will kill bacteria; that is their property as well as their purpose. That things are not this simple was shown for the first time in the case of the sulfonamides, which were then designated as bacteriostatic agents. This term however does not mean very much. If we go through a range of concentrations of any disinfectant or antiseptic we will find growth suppression, growth cessation, and bactericidal action proportional to the concentration. So bacteriostasis is not a property of a substance but of a concentration. Bigger (13) has proposed the term "bacteriofrenic action" in the case of chemotherapeutics. It may be of advantage to introduce a new word if clear insight is lacking, but there must be much more behind the concept. In itself this phenomenon may have some, perhaps great, importance with regard to the therapeutic effect. Bacteria that do not multiply can be more readily dealt with by the body (see page 429). Leaving the terms as they are, we may assume that phenomena of growth, growth suppression, and death of bacteria under the influence of chemotherapeutic substances are so complex that the comparison with antiseptics may be misleading because of the fundamental difference in their points of attack.

Smith (14) observed a lag of 30 min. in growth suppression when penicillin or chloramphenicol was added in low concentrations to *Escherichia coli* in the log phase of growth. Aureomycin acted immediately. (We regret that this time interval often is given the misleading name of "lag-time of action.") In higher concentrations penicillin and aureomycin caused a sudden drop in viable counts whereas chloramphenicol caused only bacteriostasis.

Eagle & Musselman (15) demonstrated that the bacteriostatic effect of penicillin preceded the death of sensitive bacteria; neither effect was ob-

served unless conditions were favorable for multiplication. Likewise a favorable medium was required for the recovery of the bacteria from the action of sublethal doses of penicillin. The time required before the sublethal toxic effect was demonstrable varied with the concentration of penicillin and was correlated with the rate of subsequent killing. Eagle, Fleischman & Musselman (16) thereupon studied in more detail the phenomenon of recovery from the action of penicillin. The existence of a maximal effect was demonstrated. Neither longer exposure, nor higher concentrations could enhance it (see further under CHEMOTHERAPEUTICS, PARASITE AND HOST).

The problem as to whether bacteriostasis alone is sufficient or whether an effective bactericidal result is essential in cure of human diseases by chemotherapeutic means is not yet unequivocally solved. This theme is encountered in many publications. One that deals profoundly with the question is that of Hunter (17). His conclusion in the case of bacterial endocarditis is distinctly in favor of the latter view.

As to protozoa, Hewitt *et al.* (18) reported that aureomycin acts in a concentration of 1:1000 on *Endamoeba histolytica in vitro* in from 10 to 60 min. Balamuth & Brent (19) found prodigiosin to be highly effective *in vitro* against the same organism. Amrein (20) demonstrated tyrocidin to be growth suppressing at 100 μg/ml. and lethal at 200 μg/ml. for *Trypanosoma cruzi*, these effects being independent of the presence of serum.

MICROBIAL RESPIRATION

One gets the impression that the study of the influence of chemotherapeutics on bacterial respiration, so extensive in the early days after introduction of the sulfonamides, has lost some of its original attention. The negative results which are obtained in many cases may explain this trend. Respiration among all living beings is rather uniform in its mechanism. Since chemotherapy implies specific damage to the microorganisms, it does not seem probable that the drugs will have their influence solely on this vital function, unless in special cases the mechanism differs from the general pattern.

Van Meter & Oleson (21) found aureomycin to bring respiration of rat liver homogenates to a standstill when citrate was omitted from the medium. If citrate was added, oxygen uptake started to decline after a much longer period. It is suggested that aureomycin acts through blocking some part of the Krebs cycle.

Oginsky, Smith & Umbreit (22) studied the inhibition of an oxidative reaction in a streptomycin-sensitive strain of *Escherichia coli*. This reaction was apparently the "oxaloacetate-pyruvate" condensation. This inhibition prevents a variety of substances from entering the terminal respiration system that resembles the citric acid cycle. Interrelationship between this and the preceding observation may be suggested. Smith, Oginsky & Umbreit (23) then found that streptomycin-resistant and -dependent variants

of *E. coli* are not able to effect the oxaloacetate-pyruvate condensation. Their ability to multiply in the presence of streptomycin depends on their ability to dispense with this condensation. The same investigators (24) then demonstrated that the acetate-oxaloacetate condensation cannot be the major pathway by which pyruvate enters the terminal respiration system of *E. coli*. A pyruvate-oxaloacetate reaction not forming citrate is the one that is inhibited by streptomycin. The system under investigation exists in animal tissues as well. In the intact animal, as is suggested by Umbreit & Tonhazy (25), permeability barriers will prevent streptomycin from attacking the site of this reaction (cf. p. 425), unless the barriers are overcome. Henry, Henry, Housewright & Berkman (26) also found streptomycin, in barely bacteriostatic concentrations, able to produce noncompetitive blocking of metabolism of certain carbohydrate intermediates in susceptible strains but not in resistant strains of different species of bacteria. This blocking leads to accumulation of acetate.

Sevag, Steers & Forbes (27) demonstrated the existence of a sulfonamide-resistant type of pyruvate metabolism in strains resistant to this substance. They state expressly that the resistant organisms do not by-pass pyruvate metabolism. This suggests a principal difference in sulfonamides as compared with streptomycin (cf. 23 and 26). Henry *et al.* (28) demonstrated that inhibition of growth by streptomycin runs parallel with carbohydrate utilization and oxygen consumption per cell.

In *Mycobacterium tuberculosis*, however, Andrejew (29) failed to demonstrate any influence of streptomycin on bacterial respiration, either with bacilli in the proliferating phase or in the resting condition. There was no difference between sensitive and resistant strains.

Von Grumbach (30) prepared deep tubes of agar media, mixed them with different organisms, and added different concentrations of streptomycin or penicillin. At certain concentrations of streptomycin, growth was visible in the lower but not in the upper parts of the tubes; with penicillin the opposite was the case. There were marked influences on the shift in eH at different levels as compared to drug free controls.

Osmerod (12) failed to demonstrate any influence of antrycide on respiration and glucose utilization by trypanosomes. If the concentration was increased, there might be detected an influence on respiration. This however was equal in normal and in resistant trypanosomes, which excludes respiration as the essential point of attack.

Metabolism Other than Respiration

General.—Stone (31) raised the question whether a penicillin-resistant strain might form diffusible substances that could be penicillin antagonists for a sensitive strain. He failed to demonstrate anything of the sort, so he concluded that penicillin essentially may impair the ability of the bacterium to assimilate an essential nutrient from the medium. This supports the view

of Gale & Rodwell (32) that the resistant strain has "learned" to synthesize the essential nutrient within its cells.

According to Hahn & Wisseman (33) chloramphenicol, aureomycin, terramycin, and nitroacridine 3582 suppress the ability of *Escherichia coli* to form certain adaptive enzymes. This points towards a general interference of these drugs with protein metabolism, but this effect is not per se responsible for the antibacterial properties of the substances.

Specific metabolic interference.—Research in previous years has pointed more and more towards the essential significance of impairment of bacterial anabolism by chemotherapeutics. Many enzyme systems accessible to study were shown to be unimpaired by many of the substances under discussion. It has thus been felt that probably the most specific of all processes involved in maintenance of life, i.e., the building up of the body proteins and nucleoproteins, is in some way or other hampered by substances with chemotherapeutic properties. One thing, though, will have to be kept in mind again. When an effect is observed this need not necessarily be the one effect which entirely explains the therapeutic action. Since we have no gauge of that very action, for the moment we will have to content ourselves with probabilities. We encounter divergencies as well as accordances, contradictions too being not infrequent. In the first and last case there may be three possibilities: first, the facts are not yet well established; second, research runs along the wrong track; and third, there are two or more mechanisms running parallel. Which of these might be the essential explanation of the matter is still obscure. But even if there is accordance, this does not mean that we may feel certain about more than the mere facts. This may be said to exempt the reviewer from any duty of evaluation.

On the other hand, some special enzyme systems, the utilization of essential growth factors, and the like have proven to be impaired. The aforesaid holds true in these cases also. It may be remarked in an aside that there are no allusions to the induction in the positive sense of abnormal activities or exaggeration of normal activities that may lead to "intoxication" of the microorganisms. This doubtless is the consequence of the general, but mostly subconscious, accepted way of thinking.

Interference with protein metabolism (including amino acids).—A long series of systematic work on the mode of action of sulfonamides resulted during the period of this review in three publications of Winkler, de Haan & van de Langerijt (34); de Haan, Winkler & Baljet (35), and de Haan & Winkler (36). The essential difference between competitive and noncompetitive inhibition by sulfonamides of *Escherichia coli* was their lead. A competitive inhibitor, of course, is *p*-aminobenzoic acid (PAB); noncompetitive inhibitors are methionine, xanthine, serine, thymine or pteroylglutamic acid, and valine. Each successive higher concentration of sulfonilamide demands the next noncompetitive inhibitor in the range. Sulfanilic acid, the mother substance of the sulfonamides and all tested sulfanilamide derivatives be-

have accordingly. The drug concentrations may differ, but the relative sensitivity of the systems impaired is much the same. This suggests that the mode of action of the sulfonamides is identical. Earlier observations by the same group of investigators explained the difference in activity to be due to differences in adsorption on the one hand and differences in relations to the host on the other hand. When comparing *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) with *E. coli* (35) serine falls out of the range in the case of the first organism while pteroylglutamic acid will not replace thymine for *S. aureus*. An *E. coli* mutant that requires PAB for its growth will multiply in a synthetic medium with methionine, xanthine, and thymine. Together they allow, by noncompetitive inhibition, multiplication in the highest concentration of sulfanilamide. Furthermore it was shown that the different levels of sulfonamide that are inhibited successively by the named amino acids are not the consequence of differences in sensitivity of the systems involved towards sulfanilamide but of differences in avidity to PAB.

Analysis of the sulfonamide-PAB antagonism in *Neurospora crassa* has been made by Strehler (37) with essentially the same results and but minor differences. A mutant was found which could synthesize the amino acids required up to the level of methionine.

Zalokar (38) analyzed most remarkable features in a *Neurospora* mutant that had become sulfanilamide-dependent. PAB in excess inhibited growth but could be competitively antagonized by sulfanilamide. His interpretation is that the genetic constellation which induced sulfanilamide dependence controls at the same time deleterious reactions. The complicated analysis of this concept should be consulted in the original. A related study of noncompetitive sulfanilamide antagonists in yeast has been made by Cutts & Rainbow (39).

From an elaborate series of experiments on amino acid metabolism in staphylococci and its inhibitors by Gale (40, 41, 42) and Gale & Paine (43) may be mentioned only the following conclusions. Gram positive bacteria are able to concentrate amino acids from their environment. The same ribonucleotide complex that induces gram-positiveness is the active part in this ability as well as in the following phase, i.e., formation of cellular combined glutamate. The two processes may be called *a* and *b*. Eight-hydroxyquinoline is more effective against *a* than *b*. Aureomycin and chloramphenicol are markedly more effective against *b* than *a*. Streptomycin affects *b* though only at much higher concentrations than required for growth inhibition. In chloramphenicol, growth inhibition and suppression of *b* occur in the same concentration. Penicillin has no influence on resting bacteria; after 30 to 60 min. of action on growing cultures both *a* and *b* are inhibited. The point of attack is suggested to be the nucleic acid metabolism; the suppression of amino acid assimilation is considered to be the consequence (cf. 64).

Simmonds & Fruton (44) offer the hypothesis that a peptide which contains glycine and l-leucine is synthesized by their SF strain. This peptide

markedly inhibits the action of penicillin (up to 500 units per ml.); much less inhibition is shown by the mixture of the component amino acids which does not interfere with the effect of five units per ml. The mixture of l-leucine and glycine fails to counteract penicillin because the drug inhibits the synthesis of their peptide.

Hotchkiss (45), on the other hand, observed that bacterial cells pretreated with penicillin in the presence of amino acids produce more extracellular substances containing nonamino nitrogen. Tentatively these extracellular substances are identified as polypeptides, produced instead of cellular protein in the presence of penicillin. The use of different kinds of bacteria may be one out of many explanations for the contradictory views of the two last quoted investigations.

A penicillin moiety, thioproline, effectively inhibits growth of *Escherichia coli*, Beerstecher (46) found. A large number of amino acids inhibit this growth suppression. Proline is most effective of all. Combinations of amino acids are still more inhibitory. This is not out of line with the view that penicillin prevents bacterial protein synthesis. By the way, it may be mentioned that Drea (47) could inhibit growth of *M. tuberculosis* with β-2-thienylalanine, which effect could be prevented by phenylalanine. Interference with amino acid metabolism may thus have bearing on the suppression of multiplication in tubercle bacilli as well; this highly inaccessible organism has thus been explored one step further.

As to protozoa, Ormerod (48) demonstrated that trypanocidal drugs interfere with the normal interaction of protein with ribonucleic acid in the cytoplasm.

Growth factors and chemotherapeutics.—Harrison & Clapper (49) studied the difference in vitamin requirements between a sulfonamide-sensitive strain and artificially induced resistant variants of a *Streptococcus mitis*. The resistant mutant required pteroylglutamic acid, whereas the original strain would grow in its absence. With other bacterial vitamins there was less difference. A naturally resistant *Streptococcus faecalis* required pteroylglutamic acid just as did the induced resistant *S. mitis*. This fits into the view that sulfonamides interfere somewhere in the pteroylglutamic acid-dependent anabolism. Compare, however, (35); the role of pteroylglutamic acid may be different in different species of organisms. The mechanism of sulfonamide action too may show minor or major divergence.

Pteroylglutamic acid-like activity of dehydroisoandrosterone for *S. faecalis*, *Lactobacillus casei* and sulfonamide-treated *E. coli* was seen by Gaines & Totter (50). It differs somewhat from the effects of thymine but it is of the same order of magnitude.

The matter of inhibition of the action of chloramphenicol was comprehensively studied by Woolley (51). The following may be abstracted from his publication. Phenylalanine is a noncompetitive antagonist of chloramphenicol in the case of *E. coli* in the glucose-salt medium of his experi-

ments. Then follow tyrosine and tryptophan, other amino acids being inactive. *L. casei* can be protected only by phenylalanine. The antagonism was demonstrable only in the presence of a minimal effective concentration of the antibiotic. The interpretation, however, is difficult because none of the antagonists tested was competitive. The fact that tyrosine and tryptophan partially could replace phenylalanine as a chloramphenicol antagonist for *E. coli* might be ascribed to the participation of these amino acids in phenylalanine metabolism. Structural intermediates between chloramphenicol and phenylalanine were then investigated. With progressively greater alteration of the molecule of phenylalanine, a noncompetitive and an irreversible type of antagonism with phenylalanine resulted. Most understandable results were obtained in those organisms which were dependent on phenylalanine. For the numerous details see the original. Still another system of antagonists has been comprehensively studied by Woolley (52), i.e., the antimetabolites of the precursors of riboflavin and vitamin B12. The very fundamental considerations of the author again are too extensive for review and should be read in the original, where a bibliography of previous publications will also be found.

Against *M. tuberculosis* β-3-thienylalanine and phenylalanine form a pair of antagonists as was demonstrated by Pope (53). Two other substances antagonizing phenylalanine were found by him. [See also Drea (47) and Harris & Work (150).]

A number of competitive inhibitor systems were studied by Hitchings, Elion & Falco (54) using *L. casei*. Competitive inhibition occurred between thymine and 5-aminouracil and 5-bromouracil, between folic acid and 5-nitrouracil, and finally between uracil and 5-hydroxyuracil. The activity of thymine in counteracting nitrouracil however is highly unspecific. Many pyrimidines structurally related to thymine have thymine-like action. The authors refer to the role of "secondary reversing agents," which increase the ratio of antimetabolite to metabolite required to produce a given inhibition. This phenomenon complicates interpretations. The authors warn that one must be guarded in interpreting the activities of secondary reversing agents in terms of specific enzymes and their chains of products. Other investigations too suggest that there may be some danger in interpreting too easily systems of antagonists as proof of interference with definite enzyme systems. Yegian & Long (55) (see also p. 424) demonstrated that the fact that PAB antagonizes both *p*-aminosalicylic acid (PAS) and sulfonamides does not mean that the mechanism of their action is the same because there is no cross resistance against these two drugs. Tobie & Jones (56) found that PAB antagonizes PAS in its action on *M. tuberculosis*. In other bacteria, however, PAS may be substituted for PAB so that at least it may be concluded that there is one specific difference in metabolic mechanism between tubercle bacilli and the others. In this relation the fact that protozoa, especially malarial parasites, are sensitive to sulfonamides may be of interest. This

fact suggests an identity or at least a very close relationship in certain metabolic pathways between bacteria and protozoa, the more so because PAB (cf. 148) and pteroylglutamic acid antagonize this effect. In *Plasmodium berghei*, however, Thurston (57) showed proguanil to be antagonized by PAB but not by pterolylglutamic acid; in *Plasmodium gallinaceum* the opposite was the case (see also 149).

An antagonist, the first that came to our knowledge, against streptomycin and dihydrostreptomycin has been found by Lightbown (58) to be produced by *Pseudomonas pyocyanea* (*P. aeruginosa*). Von Grumbach (59) confirmed this and found the same product to activate penicillin action (see also 30).

Special enzyme systems.—It is obvious that when specialized techniques to study given enzyme systems of microorganisms are at hand, these techniques should be applied to obtain data on the action of antibacterial substances on these systems. The interpretation of such data however can be difficult, for it may not be obvious whether the action, mostly the inhibition or suppression of the enzyme system is itself primary or whether it may be secondary to some interference with a primary affected mechanism. It should be remembered that the effect must be demonstrated to be characteristic by testing other substances both of accepted value and of no value in chemotherapy.

Henry, Henry, Housewright & Berkman (26) found that streptomycin, even in high concentration, did not interfere with catalase, carbonic anhydrase, cytochrome-cytochrome oxidase, succin-oxidase, carboxylase, urease, and trypsin. Penicillin in lethal concentration according to Bayliss, Glick & Siem (60) does not affect phosphatase and lipase.

Usnic acid is highly bacteriostatic for human tubercle bacilli. In concentrations of the same order of magnitude oxidative phosphorylation by washed residues of rat liver homogenates is "uncoupled." So Johnson, Feldott & Lardy (61) conclude that this suggests the effect on phosphorylation to be responsible for the antibiotic action. Differences in permeability (cf. 21) may explain the selective action; hence the therapeutic usefulness.

Pratt & Dufrenoy (62) by double staining of penicillin assay plates with triphenyltetrazolium chloride and with trypan blue obtained further evidence to support their earlier conclusion [Dufrenoy & Pratt (63)] that penicillin fundamentally interferes with enzyme systems involved in hydrogen transfer and dephosphorylation of complex ribonucleates. Polymyxin affects *E. coli* through essentially the same mechanism.

Also Mitchell & Moyle (64) and Gale *et al.* (40, 41, 43) (cf. section *Protein metabolism*) lay stress on the phosphoric complexes in the cell. The first authors found a particular phosphoric ester XP, that accounts for more than 20 per cent of the total phosphorous in gram positive organisms. They relate this to penicillin sensitiveness of the gram positives. Other interesting details may be read in the original.

Oginsky, Smith & Solotorovsky (65) found complete oxidation of fatty acid by *M. tuberculosis* hampered by streptomycin though the chain itself is oxidized. Streptomycin-resistant tubercle bacilli were unimpaired in their full oxidation of fatty acid. In *E. coli* stearate oxidation was inhibited, although the two organisms differ in the way they oxidize this substance. In streptomycin-resistant *E. coli* the oxidative potency for the high fatty acids is lost.

The effect of streptomycin on diamino oxidase has been studied by Owen, Karlson & Zeller (66). This oxidative enzyme is inhibited in *Mycobacterium smegmatis*, *M. avium*, *M. tuberculosis* var. *hominis*, *Pseudomonas aeruginosa* and *Micrococcus pyogenes* var. *aureus*. The enzyme is inhibited not only by streptomycin and dihydrostreptomycin but by a number of other antibiotics such as pyocyanin, streptothricin, chloramphenicol, thiosemicarbazone, promin, arcaine, and a wide variety of synthetic amines.

Gale (67) showed penicillin to be able to suppress the potency of a coliform organism to adapt to nitrate.

Finally it has been shown by Wisseman *et al.* (68) that drugs active against rickettsiae do not impair glutamate oxidation, whereas certain common enzyme inhibitors on the other hand do so.

The influence of suramin on a great number of enzyme systems had been studied by Wills & Wormall (69). Many, but by no means all, are inhibited by the drug. Where inhibition occurs, it is specific (for details see original).

Resistance to Chemotherapeutics and Mode of Action

Resistance of the infecting agent to chemotherapeutic substances had been observed by Ehrlich; the problem is as timely now as in the early days. In fact, thus far no one of the modern drugs has failed to provoke resistance and it might be asked whether it will ever be possible to find substances with chemotherapeutic effect that will not induce resistance. "Specific killing," the modern field of investigation of which chemotherapy is a branch, presumes interference with that part of the vital processes of living organisms which is more or less remote from the most fundamental features of life. In the search for the mode of action of our substances we even take it for granted that they will act on some specialized function, preferably different in host and parasite. For convenience such a function may be called a bypath. This implies a possibility for bypassing it. If this has been realized, it does not matter how, there will be resistance. So the premise of chemotherapy is the same as the one for resistance.[3] If a difference in function between a resistant and a sensitive strain in one species of an organism, or even in different species of organisms, can be outlined, we presume to have a clue to the mode of action. From this point of view, the question whether

[3] According to the reviewer's idea (151) a substance that will not induce resistance has a very slight chance to be a chemotherapeutic at all.

mutation or adaptation results in resistance is of secondary importance. In both cases only the mechanism under discussion is involved, the organism itself being unchanged in its essential characteristics.

In many cases of induced resistance, however, biological functions appear to be altered which do not as yet give us any lead of thought towards the mechanism of action. We will in those cases be forced to register the facts and nothing more.

References to observations regarding development of resistance without further elucidation of the mode of action of the drug concerned are: (70 to 77). Important data on the phenomenon of resistance as such, so close to our subject, may be drawn from these publications. For resistance in protozoa reference may be made to (78).

Under previous headings, reference has already been made to the papers of Henry *et al.* (26), Harrison & Clapper (49), and Oginsky *et al.* (65); in these investigations resistance has been considered for the purpose explained in the introduction to this section.

When resistance to one drug results in resistance to another this may point to identity or at least to very close relationship of the mode of action of the two drugs. Other explanations, it must be borne in mind, can be introduced, however (e.g., genetic linkage of two different systems, "learning" of a bypath that offers a substitute that may be of profit for two different processes).

Pansy *et al.* (79) found two different organisms which could be made resistant to aureomycin, and which also became resistant to chloramphenicol and terramycin though to somewhat lower levels. Chloramphenicol induced resistance to lower levels of aureomycin and terramycin. Clapper & Heatherman (80) found four strains of *S. mitis* to be more resistant to penicillin, streptomycin, bacitracin, and aureomycin after sulfonamide resistance was induced. They were so altered that they behaved much like *S. faecalis* (cf. 49). Acquisition of ability to grow in bile or in high concentrations of streptomycin did not alter the *S. mitis* to resemble *S. faecalis*, nor did resistance to sulfathiazole or the other antibiotics arise. Thus combined resistance in one direction need not exist in the other direction. This supports the previous supposition that combined resistance may have another explanation than identity in the mode of action.

Fusillo & Romansky (81) found cross resistance between terramycin and aureomycin; chloramphenicol may or may not join the other two drugs. Bergersen (82) saw penicillin resistance after induced aureomycin resistance.

Stone (83) found sometimes, but not always, a slight decrease in resistance to penicillin after induced bacitracin resistance; the opposite holds true as well. Pain (84), however, found penicillin and bacitracin sensitivity and resistance fairly well linked; both substances may suppress glutamic acid uptake.

Stone again (83) tried to find out whether a penicillin-resistant strain can produce diffusible substances to enable the penicillin-sensitive strain to multiply in the presence of penicillin. This not being the case, it may be considered that the resistant strain has learned to grow in the absence of an essential factor. Accordingly, Czekalowski (85) found different types of penicillin inactivators. Some organisms may produce penicillinase, which may be secreted (extracellular) or may be endocellular. Other organisms destroy the antibiotic although neither of the two types of penicillinase is demonstrated. The inactivators are supposed to play an important role in bacterial metabolism. Penicillinase-producing bacteria, on the other hand, may be as sensitive to penicillin as strains not producing the enzyme [Dietz & Bondi (86)].

Yegian & Long (55) found that there is no cross resistance between sulfonamide and PAS-resistant organisms. When sensitive to the two substances, PAB will protect them against both. This makes the authors conclude that despite the analogy suggesting competition of PAB with both substances their mode of action is different.

The prevention of streptomycin-induced resistance in the tubercle bacillus, so utterly important in therapeutics, when PAS is combined with the antibiotic was studied by Vennesland, Altona & Bloch (87). The results should be read in the original, being too extensive for brief survey.

Changes in biologic properties of resistant strains have been observed by different authors. Gezon *et al.* (88, 89) found decreased mouse virulence in beta-hemolytic streptococci resistant to aureomycin, loss of group specificity, and transient conversion from clear to green hemolysis or loss of green hemolysis when this was the only type present. In bacitracin-resistant streptococci, loss of mouse virulence could be restored by repeated mouse-passage; streptolysin "S" was produced in smaller amounts, while other streptococcal enzymes were unimpaired.

English & McCoy (90) found a *Staphylococcus aureus*, resistant to streptomycin, able to grow in a semisynthetic medium to much higher saturation than the parent strain in the same medium. Addition of biotin brought the parent strain to the same level as the resistant mutant. The references (5, 23, 27) may be once more considered in this context.

Mayer & Crane (91) observed a yellow pigment being formed by PAS-resistant strains of tubercle bacilli from this drug.

Linz & Lecocq (92) studied in more detail the "phenomenon of Welsch": when a streptomycin sensitive strain and its resistant variant (*Staphylococcus Heatley*) are grown together in a drug-free medium, after a few subcultures, invariably the resistant variant will be suppressed to the level normally present in the parent strain. The authors found that the phenomenon holds true for other strains of staphylococci, for *E. coli* and *P. aeruginosa*. If, however, a resistant and a sensitive strain from the same species but unrelated in origin are brought together, the suppression of the resistant strain may or may not take place.

PHYSICAL AND PHYSICOCHEMICAL FACTORS

It has been quoted that Johnson *et al.* (61) and Umbreit & Tonhazy (25), with usnic acid and streptomycin respectively, saw inhibition of oxidative phosphorylation and oxaloacetate-pyruvate condensation. Both groups of authors explain the difference in sensitivity of the bacteria and the intact animal cells by differences in permeability that may be chemical as well as physical in nature. Differential permeability may be the basis for selective action (61).

Surface active substances will have influence on the activity of drugs. Bailey & Cavallito (93) found aliphatic acids of 10 to 18 carbon atoms to enhance the effect of basic antibacterials (streptomycin) and decrease the effect of acidic penicillin. They explain this to be the consequence of fixation through van der Waal's bonding in or on the cell, thereby increasing the concentration of anionic groups at the site at which the antibacterial agent reacts with its substrate.

The surface active substance Triton A-20 enhances growth of a streptomycin-resistant strain of *M. tuberculosis* in low concentrations and inhibits in higher concentrations. This latter effect according to Hurwitz & Miller (94) is increased at higher pH ranges. Variations in concentration of Triton A-20, however, have no influence on streptomycin sensitivity, while changes in pH on the other hand do affect the streptomycin influence on the resistant strain of *M. tuberculosis* considerably: pH 7.0 allowed growth in 10 μg. streptomycin per ml. of medium; pH 6.5 did so at a concentration of 1000 μg. per ml. At pH 6.0, the organism grew well in the presence of 1000 μg. per ml. and with optimum growth at a concentration of 100–500 μg. Hurwitz (95) then showed the same to hold true in the case of dihydrostreptomycin. The lower the initial pH the more dependent became the drug-resistant strain. At pH 6 the organism grew poorly in low concentrations but well in high concentrations of the drug.

In heart-brain broth some constituent seems to inhibit the antibacterial activity of streptomycin as compared with that in "ordinary" nutrient broth. Linz & Lecocq (96) explain this by some inhibition of the necessary fixation of the drug on the bacteria (see also 143, 147).

An antagonism between trypaflavine (may this substance still be called a chemotherapeutic?) and cations is demonstrated by Massart & van der Stock (97). Trypaflavine is adsorbed at the protoplasmic membrane by acidic groupings, and this adsorption can be prevented by other cations, the interference being subject to the laws of Coulomb forces. The comprehensive researches of Albert *et al.* (98) and Rubbo *et al.* (99) are supplemented by the demonstration that the chemical constitution of heterocyclic bases (benzquinolines, phenanthridines, benzacridines, quinolines, and pyridines) is essential for their action on bacteria. There has to be a minimum flat area (above 28 sq. Å) or a minimum coplanar area has to be built on to the molecule before action becomes powerful. Besides, at least 50 per cent ioni-

zation at the pH of the test has to be realized. That physical factors too may be of influence on the entrance of antrycide into trypanosomes is suggested by Osmerod (12); it exerts its action in the cell and not on the surface membrane.

Brownlee *et al.* (100) found evidence that trypanocidal activity of quaternary salts of phenanthridine is to some extent dependent upon their oxidation-reduction properties; steric factors are also of importance.

Interrelationship of Chemotherapeutic Drugs, Synergism and Antagonism

Just as drugs with similar, opposite, or even seemingly unrelated action in the animal and the human being may exert unexpected pharmacological actions when combined, in the field of chemotherapy an analogous set of problems has arisen. This interrelationship of drugs is of theoretical importance and is of high practical value as well. Resistance from the very beginning has clouded our outlook in chemotherapy. Garrod's (101) gloomy outlook on chemotherapy is worth consideration. There is, however, the fact that resistance to one substance does not necessarily include resistance to any other. Since we have quite a few substances at our disposal, the idea of combining them is self-evident. Information is gradually piling up; we are still further from understanding, however, than in the matter of the mode of action of single drugs.

Initially it was presumed that combination of chemotherapeutics should be of benefit. Then came the observation that there can be an antagonistic effect in some combinations. A series of publications by Jawetz, Gunnison, Speck, Coleman, and Bruff (102 to 110) should be consulted in the original. In relation to the problem under discussion, the following may be abstracted. *In vitro*, enterococci were killed more readily by penicillin plus streptomycin than by either drug alone (105, 106). The zone phenomenon shown by penicillin was suppressed by streptomycin. Penicillin combined with chloramphenicol or with aureomycin (104, 106) had a higher bacteriostatic effect than penicillin alone, but the bactericidal rate was somewhat suppressed in the early hours of exposure (103, 106). It seemed that chloramphenicol or aureomycin antagonized the early bactericidal effect of penicillin, but that the reverse did not occur (107). In combinations of three drugs, streptomycin (or bacitracin, also synergistic with penicillin) overcame the demonstrated antagonism of chloramphenicol toward penicillin. Antagonism *in vitro* existed between aureomycin, chloramphenicol, or terramycin versus either penicillin or streptomycin acting on *Streptococcus pyogenes* or *Klebsiella pneumoniae* (103, 110). It is remarkable, however, that after interference with the early bactericidal rate, the ultimate power of killing all exposed bacteria was increased (103). This points to some highly complicated mechanism and is in some accord with the generally known view that many drugs act diphasically.

In vivo, there was definite suppression of the therapeutic effect of penicillin or of streptomycin in streptococcal infection in mice by simultaneous treatment with chloramphenicol (102, 110). Aureomycin and terramycin interfered in a much narrower range of subtherapeutic concentrations than did chloramphenicol with highly therapeutic doses of penicillin. There was no evidence of chemical interaction of the drugs combined. The antagonism is believed to be based on a modification of the infecting agent by the interfering drug. Analysis may be promising in disentanglement of the mechanism of action of the drugs.

Sulfadiazine interfered with penicillin in a remarkable way (108). When treatment in mice (*S. pyogenes*) or addition *in vitro* (*K. pneumoniae*) was started simultaneously with both drugs, there was no interference. If, however, the organisms were brought in contact with the sulfonamide previous to the introduction of penicillin, the effect of the latter in moderate concentrations was much suppressed. Whereas 95 to 100 per cent of the infected mice survived with penicillin alone, only 20 to 40 per cent survived when sulfadiazine was administered in subtherapeutic doses five to seven hours previously. The well known so-called delayed action of the sulfa drugs is brought into relationship with the characteristics of this antagonism.

Spicer (111) on the basis of her observations that combinations may complement as well as impede action, warns against haphazard combination in therapy without implicit determination of sensitivity.

Bigger (13) continued to study a wide range of synergisms and antagonisms in the case of sulfathiazole, penicillin, streptomycin, chloramphenicol, boric acid and P.A.S. on *E. coli*. Mostly there was synergism; some combinations gave antagonism, e.g., PAS versus streptomycin, chloromycetin versus sulfathiazol; the effect was only transient and feeble. Strong and permanent antagonism was shown by PAS versus sulfathiazol, the only case that he feels will have to be kept in mind in relation to human treatment.

Rees & Robson (112) found enhancement of streptomycin action, locally applied in tuberculous corneal lesions, by potassium iodide.

Despois & Pinnert (113) found staphylococci that had developed resistance to penicillin, more readily suppressed by mixtures of penicillin together with other antibiotics (streptomycin and subtilin) than by the drugs separately. This may be important from two different points of view. First, this suggests that the bacteria, though resistant to penicillin, still are under some influence of this drug; for with penicillin they are more sensitive to streptomycin or subtilin than wihout penicillin. This points to an essential feature in mechanism of action and resistance, which asks for profound exploration. Second, the practice of switching over to another antibiotic in therapy when resistance of the infecting agent has developed may not be reasonable. In such cases it may be better to continue penicillin medication and combine it with streptomycin, for example.

A well known combination of two chemotherapeutics is that of strepto-

mycin and PAS. It is already generally admitted that there will be more than simple addition of action. PAS prevents the organisms from acquiring resistance to streptomycin. Therefore the twin derivative dihydrostreptomycin paraminosalycilate (DHSM-PAS) has been synthesized. Vennesland, Altona & Bloch (87) demonstrated that resistance to DHSM-PAS could be induced nearly as easily as resistance to DHSM alone. The mixture of DHSM and PAS in a molecular ratio of 1:3 up to the moment of publication did not provoke resistance to either of the two. On the other hand, a strain made resistant to chemically bonded DHSM-PAS was resistant to DHSM alone, PAS alone, and to the mixture of both. Rees & Robson (112) also reject the chemically combined substance in favor of the mixture. This again is highly challenging: if there is a difference in the power to induce PAS resistance between the single substance and its dihydrostreptomycin compound there must be a difference, whether it be essential or only in degree, in the mode of action of the compound and the mother substance; the same then holds true for dihydrostreptomycin.

Somewhat outside the scope of synergism and antagonism of two or more drugs are the following observations: Cobalt enhances *in vitro* as well as *in vivo* the action of penicillin [Pratt & Dufrenoy (114) and Pratt, Dufrenoy & Strait (115)].

A substance, provisionally named L. substance by Bigger & Ware (116) in concentrations as low as 1:20,000 of the crude product of Lemco in which it is incorporated potentiates sulfathiazol action on *E. coli*. This, together with former observations on a similarly active product in horse hemoglobin [Winkler & Julius (117)], asks for further exploration (cf. 146).

Kayser, Besson & Pouchol (118, 119) found phenolic acid to suppress the killing action of sulfanilamide and to prolong its bacteriostatic action *in vitro*. Di- and tri-phenols showed synergism; hydroquinone most, resorcine least.

In brucellosis Renoux & Roux (120) found 2-3 dimercaptopropanol considerably synergistic with chloramphenicol or aureomycin *in vitro*. The fact that all three substances are well explored in human practice suggests trial in therapy. According to Harding & Raleigh (121) penicillin is not influenced by BAL. The substance itself is an effective brucellostatic agent.

Of purely theoretical importance for the moment is the observation of Dietz & Bondi (86, 122) that sodium azide sensitizes staphylococci to the action of penicillin without suppressing the enzyme penicillinase.

In protozoa it has been found by Greenberg (123) and Greenberg & Richeson (124) that 2,4-diamino-6,7-diphenylpyrimido (4,5-b) pyrazine, an antimalarial in chicks against *P. gallinaceum*, was markedly potentiated by sulfadiazine. This activity was partially inhibited by large oral doses of pteroyl-glutamic acid. Among a number of twenty-two compounds tested for their synergism only 2,4-diamino-5-aryloxypyrimidines were able to potentiate sulfadiazine in its activity against *P. gallinaceum* in chicks. Here

again combination of substances, even when not by themselves potent antiinfectants, may be of advantage.

To conclude, we may say that the more or less gloomy aspects, that unmistakably impend on chemotherapy, are somewhat enlightened by synergism as a phenomenon which thus highly merits to be explored extensively.

Chemotherapeutics, Parasite and Host

The classical consideration of whether the host will or will not be an "essential element" in chemotherapy is not obsolete. From the point of view of the clinician there can be no doubt as to that. One may say that it is generally taken for granted, but how the host does supplement the process is not very clear in general. Jordan *et al.* (125) failed to detect any interference of penicillin with the production of antibodies in patients treated because of pneumococcal pneumonia. In this respect penicillin may behave differently from sulfonamides.

Bliss & Todd (126) showed that one breed of mice is effectively protected by small doses of penicillin against type I pneumococcal infection. In another breed, both penicillin G and O were much less effective, ranking even well after aureomycin. If it should prove that this parallels a lower penicillin concentration in the body fluids in the latter breed, there is no problem essential to the mode of action of the chemotherapeutic. If this, however, is not the case we may be dealing with a clue to an essential problem. Eagle (127) found the dose of penicillin necessary for cure of mice and rabbits infected with penicillin-sensitive organisms to increase markedly with the number of bacteria inoculated and with the age of the infection. Eagle *et al.* (16) extended their explorations by the observation that the bactericidal effect stopped abruptly as the serum penicillin fell to an ineffective level. It took, however, a number of hours before the surviving organisms had recovered and resumed multiplication. This time was much longer than *in vitro*. To establish this grade of damage to the organisms took one hour and a barely "effective" concentration of penicillin; longer exposure or higher drug concentration were of no stronger effect. Pretreatment with penicillin of group B streptococci enhanced their susceptibility to the host in such a way that they continued to die after inoculation into mice though *in vitro* recovery would long before have taken place. Also one dose of penicillin sufficient to keep an effective level in the bloodstream for only one half hour sufficed to make the bacteria decrease in number in the animals' tissue just as if treatment had been continued. There seems to be something changed in the microorganism beyond mere hampering of growth.

That the host may intervene by changing the drugs administered, a problem very early recognized is once more demonstrated by Francis & Spinks (128) with promine, sulfetron, and diasone. Their action goes no

further than corresponds to the liberation of diaminodiphenyl sulfone; the view is held that there will be no essential difference between these drugs and the mother substance. Their difference in toxicity is merely apparent and corresponds to the level of splitting off of the diphenylsulfone.

On the other hand, the host may protect an infectious agent against a drug that per se is active. This is suggestively made into an experiment by Magoffin & Spink (129). Streptomycin alone or in combination with other agents failed to eliminate *Brucella* when the bacteria were harbored intracellularly. It may be worthwhile to go deeper into this matter; the idea is obvious and well known with viruses but our data are not so numerous.

The order of activity of phenanthridine compounds against *T. rhodesiense* and *T. congolense in vitro* and *in vivo* is the same according to Lock (130); the effective concentrations *in vivo*, however, appear much lower than the effective concentrations *in vitro*.

Josephson *et al.* (131) found a metabolite of pamaquine in the chicken to be about 16-fold as active on plasmodia *in vitro* as pasmaquine itself. The methemoglobin-producing properties are enhanced to the same degree. By spectro-analysis of the hitherto unknown substance its structure is approached.

Curd & Davey (132) suppose the remarkable prolonged protection of antrycide against trypanosomal infection (one dose for several weeks) to be due to a reservoir at the site of the injection from which absorption takes place slowly and not to persistence of the drug in the body in the usually accepted sense of the term.

Miscellanea

A remarkable observation made by several authors (16, 63, 105, 114, 133) is the so called zone phenomenon. Penicillin action is maximal at a certain concentration above and below which inhibition of cellular activity is less pronounced. Likewise, the bactericidal effect is maximal in a certain concentration of penicillin; higher as well as lower concentrations allow somewhat more organisms to survive. The most extensive study has been performed by Eagle (133). His considerations on the relation to the mechanism of action of penicillin will have to be consulted in the original, being too comprehensive to summarize.

This zone phenomenon, however, is apparent only in some strains of microorganisms and is by no means universal (e.g., in enterococci but not commonly in other streptococci). It may perhaps be of interest to correlate this phenomenon with that of absorption of penicillin by resting staphylococci as observed by Maass & Johnson (134). The drug remains bound during subsequent multiplication of the bacteria in penicillin-free medium. The uptake of penicillin is unchanged in amount but the rate in low concentrations is much lower, many hours being required in that case. If the microorganisms grow in low concentrations, the uptake continues which

suggests that the penicillin-binding component is synthesized more rapidly than the building-up of cellular substance proper. Using tagged penicillin Rowley *et al.* (135) could perfectly confirm these observations. This may be brought up in connection with the still not entirely elucidated question of whether cell division is or is not an essential factor in the bactericidal effectiveness of penicillin (compare earlier views regarding the sulfonamides).

Fixation of streptomycin by "ordinary" bacteria and by *M. tuberculosis* is demonstrated by Linz *et al.* (136, 137, 138). They suggest the process to be diphasic: in the first phase, the interaction is reversible; in the second, it is not. The uptake in the first phase augments gradually, independent of the temperature, but proportional to the concentration in the medium, and is completed to the irreversible state after four hours. A suggestion that diphasic action may be a more general phenomenon than with chemotherapeutics alone is found in the observation made by Van Eseltine & Rahn (139), that bacteriostasis by mercuric dichloride requires higher concentrations when the temperature is heightened but that bactericidal concentrations can be diminished when the temperature is raised. This even may suggest that there are separate points of attack for both effects.

Foley & Winter (140) found penicillin G to have a deleterious effect on *Candida albicans* infections of the chorio-allantoic membrane of 10-day chick embryos. The drug seems to stimulate the microorganism *in vitro*. Moore (11) found aureomycin to be growth promoting for *C. albicans* and judges the drug to be contra-indicated in pulmonary mycosis caused by the organism.

Von Jirovec (141) investigated the influence of a number of antibiotics on protozoa. Penicillin and streptomycin are innocuous, tyrothricin and terramycin toxic, and the other ones tested can be classified in between (see also under *Growth factors*).

According to Linz (142) streptomycin combines (see also 136, 137, 138, 143) with cell constituents to form a complex as it does with albumen in serum of man and animal. This complex, remarkably, remains active. Suramin behaves somewhat like streptomycin [Wilson & Wormall (144)].

Special attention may be drawn to the work of Rutten, Winkler & de Haan (145) for apparently these authors introduce a new way of thinking. When *E. coli B* is brought into high concentrations of sulfanilamide and at the same time is "protected" against its action with the four noncompetitive antagonists (34, 35, 36) the intracellular multiplication of the bacteriophages T_2, T_4, and T_6 is completely inhibited. In the reviewer's opinion a hitherto unforseen principle is thus introduced in chemotherapy. The authors demonstrated that the phages are not affected themselves by the drug but that the enzyme system in the host (*E. coli*), upon which the parasite (the phage) has to rely, is blocked, with resulting failure of multiplication of the phage. Not only in chemotherapy of virus diseases but also in the case of infections of metazoal hosts by microorganisms this lead

may be of value. If an antibacterial drug pharmacologically too toxic for application may be tolerated by administering an "antidote," not antagonizing its point of attack in the host but compensating for the temporary suppression of the attacked system, chemotherapy may be performed. Not, however, until we have penetrated very much deeper into the mode of action of chemotherapeutics, not only on the microorganisms but on the hosts as well, will we be able to go as far as suggested.

LITERATURE CITED

1. Hotchkiss, R. D., *Ann. Rev. Microbiol.*, **2**, 183–209 (1948)
2. Cutting, W. C., *Ann. Rev. Microbiol.*, **3**, 137–54 (1949)
3. Miller, C. P., and Bohnhoff, M., *Ann. Rev. Microbiol.*, **4**, 201–16 (1950)
4. Peck, R. L., and Lyons, J. E., *Ann. Rev. Biochem.*, **20**, 367–406 (1951)
5. Fleming, A., Voureka, A., Kramer, I. R. H., and Hughes, W. H., *J. Gen. Microbiol.*, **4**, 257–69 (1950)
6. Hunter, M. E., Mudd, S., and Woodburn, M. A., *J. Bact.*, **60**, 315–20 (1950)
7. Crofts, J. E., and Evans, D. G., *Brit. J. Exptl. Path.*, **31**, 550–61 (1950)
8. Johnstone, K. I., Crofts, J. E., and Evans, D. G., *Brit. J. Exptl. Path.*, **31**, 562–65 (1950)
9. Benda, R., and Urquia, D. A., *Rev. tuberc. (Paris)*, **14**, 343–47 (1950)
10. Linz, R., *Compt. rend. soc. biol.*, **145**, 463–64 (1951)
11. Moore, M., *J. Lab. Clin. Med.*, **37**, 703–12 (1951)
12. Ormerod, W. E., *Brit. J. Pharmacol.*, **6**, 325–33 (1951)
13. Bigger, J. W., *Lancet*, **II**, 46–50 (1950)
14. Smith, C. H., *J. Gen. Microbiol.*, **5**, xvii (1951)
15. Eagle, H., and Musselman, A. D., *J. Bact.*, **58**, 475–90 (1949)
16. Eagle, H., Fleischman, R., and Musselman, A. D., *Ann. Internal Med.*, **33**, 544–71 (1950)
17. Hunter, T. H., *J. Am. Med. Assoc.*, **144**, 524–27 (1950)
18. Hewitt, R., Wallace, W., and White, E., *Science*, **112**, 144–46 (1950)
19. Balamuth, W., and Brent, M. M., *Proc. Soc. Exptl. Biol. Med.*, **75**, 374–78 (1950)
20. Amrein, Y. U., *Proc. Soc. Exptl. Biol. Med.*, **76**, 744–46 (1951)
21. Van Meter, J. C., and Oleson, J. J., *Science*, **113**, 273 (1951)
22. Oginsky, E. L., Smith, P. H., and Umbreit, W. W., *J. Bact.*, **58**, 747–59 (1949)
23. Smith, P. H., Oginsky, E. L., and Umbreit, W. W., *J. Bact.*, **58**, 761–67 (1949)
24. Umbreit, W. W., Smith, P. H., and Oginsky, E. L., *J. Bact.*, **61**, 595–604 (1951)
25. Umbreit, W. W., and Tonhazy, N. E., *J. Bact.*, **58**, 769–76 (1949)
26. Henry, J., Henry, R. J., Housewright, R. D., and Berkman, S., *J. Bact.*, **56**, 527–39 (1948)
27. Sevag, M. G., Steers, E., and Forbes, M., *Arch. Biochem.*, **25**, 185–90 (1950)
28. Henry, R. J., Housewright, R. D., and Berkman, S., *J. Bact.*, **57**, 447–51 (1949)
29. Andrejew, A., *Compt. rend. soc. biol.*, **144**, 778–80 (1950)
30. Von Grumbach, A., *Schweiz. Z. Path. u. Bakt.*, **13**, 586–93 (1950)
31. Stone, J. L., *Science*, **113**, 493–94 (1951)
32. Gale, E. F., and Rodwell, A. W., *J. Gen. Microbiol.*, **3**, 127–42 (1949)
33. Hahn, F.E., and Wisseman, C.L., Jr., *Proc. Soc. Exptl. Biol. Med.*, **76**, 533–35 (1951)
34. Winkler, K. C., de Haan, P. G., and van de Langerijt, J., *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **15**, 129–35 (1949)
35. de Haan, P. G., Winkler, K. C., and Baljet, H., *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **15**, 136–42 (1949)
36. de Haan, P. G., and Winkler, K. C., *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **17**, 107–18 (1951)
37. Strehler, B. L., *J. Bact.*, **59**, 105–11 (1950)
38. Zalokar, M., *J. Bact.*, **60**, 191–203 (1950)
39. Cutts, N. S., and Rainbow, C., *J. Gen. Microbiol.*, **5**, 860–68 (1951)
40. Gale, E. F., *Biochem. J.*, **48**, 286–90 (1951)
41. Gale, E. F., *Biochem. J.*, **48**, 290–97 (1951)

42. Gale, E. F., *Biochem. J.*, **47**, xxvi (1950)
43. Gale, E. F., Paine, T. F., *Biochem. J.*, **48**, 298–300 (1951)
44. Simmonds, S., and Fruton, J. S., *Science*, **111**, 329–31 (1950)
45. Hotchkiss, R. D., *J. Exptl. Med.*, **91**, 351–64 (1950)
46. Beerstecher, E., Jr., *Proc. Soc. Exptl. Biol. Med.*, **73**, 200–1 (1950)
47. Drea, W. F., *J. Bact.*, **56**, 257–58 (1948)
48. Ormerod, W. E., *Brit. J. Pharmacol.* **6**, 334–41 (1951)
49. Harrison, V. M., and Clapper, W. E., *Proc. Soc. Exptl. Biol. Med.*, **74**, 857–60 (1950)
50. Gaines, D. S., and Totter, J. R., *Proc. Soc. Exptl. Biol. Med.*, **74**, 559–61 (1950)
51. Woolley, D. W., *J. Biol. Chem.*, **185**, 293–305 (1950)
52. Woolley, D. W., *J. Exptl. Med.*, **93**, 13–24 (1951)
53. Pope, H., *J. Bact.*, **58**, 223–28 (1949)
54. Hitchings, G. H., Elion, G. B., and Falco, E. A., *J. Biol. Chem.*, **185**, 643–49 (1950)
55. Yegian, D., and Long, R. T., *J. Bact.*, **61**, 747–49 (1951)
56. Tobie, W. C., and Jones, M. J., *J. Bact.*, **57**, 573 (1949)
57. Thurston, J. P., *Lancet*, **II**, 438 (1950)
58. Lightbown, J. W., *Nature*, **166**, 356–57 (1950)
59. Von Grumbach, A., *Schweiz. Z. Path. u. Bakt.*, **14**, 334–37 (1951)
60. Bayliss, M., Glick, D., and Siem, R. A., *J. Bact.*, **55**, 307–16 (1947)
61. Johnson, R. B., Feldott, G., and Lardy, H. A., *Arch. Biochem.*, **28**, 317–23 (1950)
62. Pratt, R., and Dufrenoy, J., *J. Bact.*, **57**, 9–12 (1949)
63. Dufrenoy, J., and Pratt, R., *J. Bact.*, **56**, 99–105 (1948)
64. Mitchell, P., and Moyle, J., *Nature*, **166**, 218–20 (1950)
65. Oginsky, E. L., Smith, P. H., and Solotorovsky, M., *J. Bact.*, **59**, 29–44 (1950)
66. Owen, C. A., Karlson, A. G., and Zeller, E. A., *J. Bact.*, **62**, 53–62 (1951)
67. Gale, E. F., *Brit. J. Exptl. Path.*, **30**, 356–64 (1949)
68. Wisseman, C. L., Jr., Jackson, E. B., Hahn, F. E., Ley, A. C., and Smadel, J. E., *J. Immunol.*, **67**, 123–35 (1951)
69. Wills, E. D., and Wormall, A., *Biochem. J.*, **47**, 158–70 (1950)
70. Hamburger, M., and Berman, J. R., *J. Clin. Invest.*, **29**, 630–37 (1950)
71. Gocke, T. M., and Finland, M., *Proc. Soc. Exptl. Biol. Med.*, **74**, 824–29 (1950)
72. Meads, M., Harris, C. M., Haslam, N. M., and Cline, W. A., *J. Clin. Invest.*, **29**, 1474–79 (1950)
73. Barber, M., *J. Gen. Microbiol.*, **3**, 274–81 (1949)
74. Yegian, D., and Budd, V., *J. Bact.*, **61**, 167–70 (1951)
75. English, A. R., and McCoy, E., *J. Bact.*, **61**, 51–56 (1951)
76. English, A. R., and Gelwicks, P. C., *Antibiotics and Chemotherapy*, **1**, 118–24 (1951)
77. Newcombe, H. B., and McGregor, J., *J. Bact.*, **62**, 539–44 (1951)
78. Edeson, J. F. B., and Field, J. W., *Brit. Med. J.*, **I**, 147–51 (1950)
79. Pansy, F. E., Khan, P., Pagano, J. F., and Donovick, R., *Proc. Soc. Exptl. Biol. Med.*, **75**, 618–20 (1950)
80. Clapper, W. E., and Heatherman, M. E., *Proc. Soc. Exptl. Biol. Med.*, **73**, 153–56 (1950)
81. Fusillo, M. H., and Romansky, M. J., *Antibiotics and Chemotherapy*, **1**, 107–9 (1951)
82. Bergersen, F., *Nature*, **167**, 995–96 (1951)

83. Stone, J. L., *J. Infectious Diseases*, **85**, 91–96 (1949)
84. Paine, T. F., Jr., *J. Bact.*, **61**, 259–60 (1951)
85. Czekalowski, J. W., *J. Path. Bact.*, **62**, 85–102 (1950)
86. Dietz, C. C., and Bondi, A., *J. Bact.*, **55**, 842–47 (1947)
87. Vennesland, K., Altona, J., and Bloch, R. G., *Proc. Soc. Exptl. Biol. Med.*, **75**, 436–38 (1950)
88. Gezon, H. M., and Fasan, D. M., *Proc. Soc. Exptl. Biol. Med.*, **73**, 10–15 (1950)
89. Gezon, H. M., Fasan, D. M., and Collins, G. R., *Proc. Soc. Exptl. Biol. Med.*, **74**, 505–9 (1950)
90. English, A. R., and McCoy, E., *J. Bact.*, **62**, 19–26 (1951)
91. Mayer, R. L., and Crane, C., *Nature*, **165**, 37 (1950)
92. Linz, R., and Lecocq, E., *Compt. rend. soc. biol.*, **145**, 143–46 (1951)
93. Bailey, J. H., and Cavallito, C. J., *J. Bact.*, **60**, 269–74 (1950)
94. Hurwitz, C., and Miller, J. B., *Am. Rev. Tuberc.*, **62**, 91–98 (1950)
95. Hurwitz, C., *Am. Rev. Tuberc.*, **63**, 568–78 (1951)
96. Linz, R., and Lecocq, E., *Compt. rend. soc. biol.*, **144**, 1274–75 (1950)
97. Massart, L., and van der Stock, J., *Nature*, **165**, 852–53 (1950)
98. Albert, A., Rubbo, S. D., and Burvill, M. I., *Brit. J. Exptl. Path.*, **30**, 159–75 (1949)
99. Rubbo, S. D., Albert, A., and Gibson, M. I., *Brit. J. Exptl. Path.*, **31**, 425–41 (1950)
100. Brownlee, G., Goss, M. D., Goodwin, L. G., Woodbine, M., and Walls, L. P., *Brit. J. Pharmacol.*, **5**, 261–76 (1950)
101. Garrod, L. P., *Brit. Med. J.*, **I**, 205–10 (1951)
102. Jawetz, E., and Speck, R. S., *Proc. Soc. Exptl. Biol. Med.*, **74**, 93–96 (1950)
103. Gunnison, J. B., Coleman, V. R., and Jawetz, E., *Proc. Soc. Exptl. Biol. Med.*, **75**, 549–52 (1950)
104. Jawetz, E., Gunnison, J. B., and Coleman, V. R., *Science*, **111**, 254–56 (1950)
105. Jawetz, E., and Gunnison, J. B., *J. Lab. Clin. Med.*, **35**, 488–96 (1950)
106. Gunnison, J. B., Jawetz, E., and Coleman, V. R., *J. Lab. Clin. Med.*, **36**, 900–11 (1950)
107. Jawetz, E., Gunnison, J. B., Speck, R. S., and Coleman, V. R., *Arch. Internal Med.*, **87**, 349–59 (1951)
108. Speck, R. S., Jawetz, E., and Gunnison, J. B., *Arch. Internal Med.*, **88**, 168–74 (1951)
109. Gunnison, J. B., Speck, R. S., Jawetz, E., and Bruff, S. A., *Antibiotics and Chemotherapy*, **1**, 259–66 (1951)
110. Jawetz, E., Gunnison, J. B., and Speck, R. S., *Am. J. Med. Sci.*, **222**, 404–12 (1951)
111. Spicer, S., *J. Lab. Clin. Med.*, **36**, 183–91 (1950)
112. Rees, R. J. W., and Robson, J. M., *Science*, **112**, 790–91 (1950)
113. Despois, R., and Pinnert, S., *Compt. rend. soc. biol.*, **144**, 23–25 (1950)
114. Pratt, R., and Dufrenoy, J., *J. Bact.*, **55**, 727–37 (1947)
115. Pratt, R., Dufrenoy, J., and Stratt, L. A., *J. Bact.*, **55**, 75–77 (1947)
116. Bigger, J. W., and Ware, G. C., *Lancet*, **II**, 427–33 (1950)
117. Winkler, K. C., and Julius, H. W., *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **8**, 86–96 (1942)
118. Kayser, F., Besson, S., and Pouchol, J., *Compt. rend. soc. biol.*, **145**, 421–22 (1951)

119. Kayser, F., Besson, S., and Pouchol, J., *Compt. rend. soc. biol.*, **145**, 423–24 (1951)
120. Renoux, G., and Roux, J., *Ann. inst. Pasteur*, **80**, 189–95 (1951)
121. Harding, H. B., and Raleigh, G. W., *J. Infectious Diseases*, **86**, 88–94 (1950)
122. Dietz, C. C., and Bondi, A., *J. Bact.*, **55**, 849–54 (1947)
123. Greenberg, J., *J. Pharmacol. Exptl. Therap.*, **97**, 484–87 (1949)
124. Greenberg, J., and Richeson, E. M., *J. Pharmacol. Exptl. Therap.*, **99**, 444–49 (1950)
125. Jordan, W. S., Badger, G. F., and Dingle, J. H., *J. Clin. Invest.*, **29**, 161–68 (1950)
126. Bliss, E. A., and Todd, P. H., *J. Bact.*, **58**, 61–72 (1949)
127. Eagle, H., *J. Exptl. Med.*, **90**, 595–607 (1949)
128. Francis, J., and Spinks, A., *Brit. J. Pharmacol.*, **5**, 565–83 (1950)
129. Magoffin, R. L., and Spink, W. W., *J. Lab. Clin. Med.*, **37**, 924–30 (1951)
130. Lock, J. A., *Brit. J. Pharmacol.*, **5**, 398–408 (1950)
131. Josephson, E. S., Taylor, D. J., Greenberg, J., and Ray, A. P., *Proc. Soc. Exptl. Biol. Med.*, **76**, 700–3 (1951)
132. Curd, F. H. S., and Davey, D. G., *Brit. J. Pharmacol.*, **5**, 25–32 (1950)
133. Eagle, H., *J. Bact.*, **62**, 63–68 (1951)
134. Maass, E. A., and Johns[illegible]. M. J., *J. Bact.*, **58**, 361–66 (1949)
135. Rowley, D., Cooper, P. D., and Roberts, P. W., *Biochem. J.*, **46**, 157–61 (1950)
136. Linz, R., Lane, J., and van Damme, O., *Compt. rend. soc. biol.*, **144**, 307–8 (1950)
137. Linz, R., and Lecocq, E., *Compt. rend. soc. biol.*, **144**, 308–9 (1950)
138. Linz, R., and Arnaud, Y., *Compt. rend. soc. biol.*, **144**, 1556–58 (1950)
139. Van Eseltine, W. P., and Rahn, O., *J. Bact.*, **57**, 547–54 (1949)
140. Foley, G. E., and Winter, W. D., *J. Infectious Diseases*, **85**, 268–74 (1949)
141. Von Jirovec, O., *Schweiz. Z. Path. u. Bakt.*, **14**, 653–66 (1951)
142. Linz, R., *Compt. rend. soc. biol.*, **144**, 309–11 (1950)
143. Linz, R., and Lecocq, E., *Compt. rend. soc. biol.*, **144**, 991–93 (1950)
144. Wilson, E. J., and Wormall, A., *Biochem. J.*, **45**, 224–31 (1949)
145. Rutten, F. J., Winkler, K. C., and de Haan, P. G., *Brit. J. Exptl. Path.*, **31**, 369–79 (1950)
146. Harper, G. J., and Cawston, W. C., *J. Path. Bact.*, **57**, 59–66 (1945)
147. Linz, R., and Lecocq, E., *Compt. rend. soc. biol.*, **144**, 993–94 (1950)
148. Thurston, J. P., *Brit. J. Pharmacol.*, **5**, 409–16 (1950)
149. Terzian, L. A., Stahler, N., and Weathersby, A. B., *J. Infectious Diseases*, **84**, 47–55 (1949)
150. Harris, J. I., and Work, T. S., *Biochem. J.*, **46**, 190–95 (1950)
151. Julius, H. W., *Koninkl. Nederland. Akad. Wetenschap.*, 140–163 (1950)

BIOLOGICAL ACTIVITIES OF METABOLITE ANALOGUES[1]

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Introduction

Although competitive inhibition of enzymatic reactions by compounds structurally related to the substrate had been known for some time, Woods' discovery of the competitive relationship of *p*-aminobenzoic acid (PAB) to sulfonamides in preventing the bacteriostatic action of the drugs (1, 2) initiated a broad field of research concerning the biological effects of compounds structurally related to known metabolites (3). More than a decade of intensive research has been spent in a search for antimetabolites as possible chemotherapeutic agents. Whereas this approach to chemotherapy has not as yet reached fruition, antimetabolites have been synthesized which are effective in preventing or curing certain infections in a host, but none of the antimetabolites discovered in this way has found general use in the treatment of infections in human beings.

Even though antimetabolites as chemotherapeutic agents have not made outstanding progress, the use of the compounds in studying metabolism has afforded not only a new method for the study of biochemistry but has thrown some light upon the difficulties encountered in the attempts to design chemotherapeutic agents as analogues of metabolites (4).

Theoretical Considerations

Inhibitory analogues of metabolites usually have structurally resembled the metabolite. Modifications of the structure of a metabolite do not necessarily lead to an analogue inhibiting the utilization of a metabolite. Usually an inert compound and, in a few cases, a compound possessing the biological activity of the metabolite are found. In the latter case, the analogue usually is much less effective than the metabolite and replaces the function of the metabolite only at relatively much higher concentrations. Of the analogues which have inhibitory properties, the inhibitory action of some may not be prevented by the metabolite which the analogue resembles structurally. In such cases, it is doubtful that the analogue is actually inhibiting the function of the particular metabolite, but more likely is exerting an effect on a different system. In the absence of the ability of a metabolite to prevent the inhibitory action of a compound of analogous structure, such a compound should not be considered to be an inhibitory analogue of the metabolite unless other data indicate the inhibitor is noncompetitively preventing a function of the metabolite.

[1] The survey of the literature pertaining to this review was concluded in February, 1952.

An analogue of a metabolite usually exerts its biological effect by combining with an enzyme which normally reacts with the metabolite to form a complex. Certain functional groups, steric factors, and configuration determine the ability of an analogue to combine with a particular enzyme. If the enzyme converts the analogue to a product which replaces the normal enzymatic product derived from the metabolite, the analogue possesses biological activity similar to that of the metabolite. In most cases, however, either the complex does not undergo further changes except dissociation into component parts or the enzymatic action on the analogue results in a product which cannot replace the normal product. In these cases, the analogue prevents the normal utilization of the metabolite by the enzyme system and thereby exerts an inhibitory effect upon the normal enzymatic process.

The response of a biological system inhibited by an analogue of a metabolite is related to the concentration of a particular enzyme metabolite complex. The concentration of this complex is approximately determined by a ratio of concentrations of analogue to metabolite rather than by the absolute concentration of the inhibitor. Thus, during a defined experimental period, essentially a constant ratio (the inhibition index) of concentrations of analogue to metabolite is required for a defined degree of inhibition, when only the concentrations of analogue and metabolite are varied.

Although the inhibitory effects of antimetabolites can be prevented in a competitive fashion over wide ranges in concentrations by the particular metabolites, other substances may also affect such inhibitions (5, 6). These reversing agents include (*a*) substances increasing the concentration of the metabolite synthesized by the biological system, (*b*) the product or its equivalent of the inhibited enzyme system, (*c*) substances exerting a "sparing effect" on the amount of this product necessary for the defined biological response, (*d*) compounds which increase the effective enzyme concentrations, and (*e*) substances which increase or decrease the rate of destruction of the inhibitor or metabolite. Inhibition indices determined at sufficiently high concentrations of the metabolite and its antagonist are not affected by reversing agents of types (*a*) and (*e*); for example, in the presence of sufficiently high concentrations of metabolite supplied to the biological system, the contribution to that concentration resulting from increased synthesis of the metabolite by the biological system becomes insignificantly small. Similarly, the destructive factors can be "diluted out."

If the product of such an inhibited biological system can be and is supplied to the biological system, the inhibited enzyme system is no longer essential for the biological system, and the analogue either becomes ineffective as an inhibitor or at higher concentrations inhibits the utilization of the metabolite in still another enzyme system. In the latter case, a new inhibition index corresponding to this enzyme system is obtained.

If a substance added exogenously decreases the requirement of the biological system for the product of an enzyme system inhibited by an analogue of a metabolite, a decrease in the rate of synthesis of the product by

increasing the ratio of concentrations of analogue to metabolite is necessary to maintain a defined degree of inhibition of the biological system. Also, an increase in the ratio of concentrations of analogue to metabolite is necessary to maintain a defined degree of inhibition when a substance increasing the effective enzyme concentration is supplied to the biological system. The two types of reversing agents act synergistically but do not replace the product effect. Also, data which reflects dissociation constants of the enzyme systems can differentiate these effects from those of the product of the inhibited enzyme system.

In view of these theoretical considerations, it is not surprising that most analogues inhibitory for organisms grown in minimal media are either less effective or inactive against the organisms *in vivo* or in complex media. Since similar metabolic pathways exist in both infecting organisms and the host, it is interesting that some metabolite analogues are specific in inhibiting only the infecting organism. While such results can often be explained on the basis of different affinities of the analogue for the particular enzyme systems of each organism, the ability of the host cell to supply the infecting organism with the end-products of the inhibited functions of the metabolite must be limited in some manner. The mode of action of sulfonamide is interesting in this respect.

B-Vitamins

p-Aminobenzoic acid.—Increasing concentrations of sulfonamide apparently decrease the rate of conversion of PAB to a coenzyme form, for which various apoenzymes compete. As the quantitative requirements for the factor vary from one system to another, one particular system becomes limiting. If the product of this system can be and is supplied to the organism, the inability of the enzyme system to function does not affect the organism in its growth responses. However, still higher concentrations of sulfonamide may reduce the formation of a cofactor to such an extent that still another system becomes limiting. If the product of this next enzyme system is supplied to the organism, the inhibition to the organism is again prevented. Thus, a series of end-products, exerting such a sparing effect on the amount of a cofactor necessary for a defined biological response, exerts its effects in a defined order. As the ratio of the concentrations of sulfonamide to PAB determines the rate of coenzyme synthesis, the end-products exert their effects by increasing the ratio, the inhibition index, necessary for a defined inhibition. For *Escherichia coli*, methionine, then purines, then serine, and finally thymidine or thymine exert such an effect, and each is dependent upon the presence of all of those preceding it in order to exert its effect (7, 8). Homocysteine does not take the place of methionine,5-amino-4-imidazolecarboxamide which accumulates in the medium under these conditions (9, 10), and glycine does not replace serine (8, 11) nor does serine replace glycine as a precursor of aminoimidazolecarboxamide under these conditions (12). Thus, the incorporation of a single carbon unit into these compounds appears to be

the function of PAB. In other organisms, the order in which the various products become limiting may vary (13).

Since folic acid has been implicated in the same reactions (14 to 22) and contains PAB as a part of its structure, the interrelationship of these factors at first appears to be that PAB is simply a precursor of folic acid which in turn is converted into a coenzyme form. However, only a few organisms which require PAB for growth are able to utilize folic acid, and some of these do not utilize folic acid as efficiently as PAB (23 to 26), or utilize it in an indirect manner (27). On the other hand, in organisms which do not utilize folic acid, sulfonamides prevent the biosynthesis of substances possessing the biological activity of folic acid (28). It appears likely that for organisms requiring folic acid for growth, the same coenzyme(s) are synthesized from folic acid as are synthesized from PAB in organisms requiring the latter but not utilizing folic acid; consequently, either folic acid does not penetrate to its site of action in the latter variety of organisms, or folic acid is not an intermediate in the biosynthesis of the coenzyme form of PAB in these organisms. The recent characterization of a new form of folic acid, folinic acid (citrovorum factor) (29 to 33), which is more active than folic acid in reversing a folic acid antagonist for *Lactobacillus casei* and in promoting the growth of *Leuconostoc citrovorum* tend to support the latter possibility. For at least one organism, folinic acid but not folic acid is able to replace PAB (34). However, for most of the other organisms of this type including *E. coli*, folinic acid does not represent a form of folic acid which can be utilized in place of PAB. In all cases where folic acid or folinic acid replace PAB the toxicity of sulfonamides is completely prevented in a noncompetitive manner. Also, the development of resistance to sulfonamides in many cases can be correlated with ability of the organism to utilize folic acid (35). Thus, a form of folic acid utilizable in such a manner by pathogenic organisms inhibited *in vivo* by sulfonamides does not appear to exist in adequate concentrations if at all at the site of the infection. It is possible that the conversion of PAB to the coenzyme form involves derivatives which either are not permeable to the cell walls or are destroyed in penetrating the cell walls. In either case, existence of such a factor in the host cell would not affect the inhibition of the invading organism by sulfonamides.

Similarly, the host cell must not be able to furnish the invading organism with all of the end-products of PAB functioning. Again, permeability factors or destruction of the products passing through the cell walls may play a role. In any event, chemotherapeutic antimetabolites must prevent or markedly inhibit enzymatic processes, the products of which cannot be supplied in adequate concentrations by the host cells.

The rate of excretion of an antimetabolite may also greatly affect its chemotherapeutic effect. The ability to maintain adequate concentrations of sulfonamides is illustrated by the effect of PAB on *Plasmodium berghei* in mice treated with sulfadiazine. High concentrations (four times that of sulfonamide) of PAB prevent the chemotherapeutic effect, but after several

days, the excess PAB is excreted, leaving enough of the sulfonamide in the blood to inhibit the organism. Thus, the metabolite is excreted at a faster rate than the antimetabolite in this case. For *Plasmodium gallinaceum*, PAB prevents the chemotherapeutic effects of 10 times its concentration of sulfadiazine (36), so that the effect of more rapid excretion of PAB is not so pronounced.

The exact mechanism by which PAB is incorporated into folic acid is still unknown; however, 7-hydroxyfolic acid, which has about the same effect as folic acid on *Streptococcus faecalis R* but has no effect on *L. casei* or *L. citrovorum* (37), has been proposed as an intermediate in the biosynthesis of folic acid. The growth promoting pterins are divided into two groups, one of which requires N-glycoside formation for activation. It is proposed that the N-glycoside of 2-amino-4,7-dihydroxypterinaldehyde-(6) reacts with *p*-aminobenzoylglutamic acid which is converted to the hydroxyfolic acid. Since sulfathiazole inhibits the utilization of this aldehyde, it was proposed that sulfonamides exert their effect at the stage of the combination with *p*-aminobenzoylglutamic acid (38, 39, 40). However, *p*-aminobenzoylglutamic acid does not effectively reverse the toxicity of sulfonamides for most organisms.

The potentiation of the antibacterial action of sulfathiazole by glutamine presumably results from the involvement of some glutamyl derivative in the metabolism of PAB (41).

The bacteriostatic activity of *p*-aminosalicylic acid for tubercle bacilli is prevented by PAB (42 to 45). Thiamine also inhibits the bacteriostatic action of *p*-aminosalicylic acid on virulent human tubercle bacteria *in vitro*, but does not exert such an effect in infected guinea pigs (46). Pantothenic acid reverses the toxicity of both salicylic acid and *p*-aminosalicylic acid, and biotin prevents the inhibitory action of *p*-aminosalicylic acid for tubercle bacilli (43). Similar results were previously obtained with 2-chloro-4-aminobenzoic acid, the toxicity of which is competitively reversed by PAB and is also reversed by pantothenic acid (47), glutamic acid, and thiamine for *E. coli* (4). During short incubations, the last three compounds do not affect the toxicity appreciably and apparently exert their effects indirectly.

After reversal of the toxicity of 2-chloro-4-aminobenzoic acid for *E. coli* by methionine, containing C^{14} in the methyl group, methionine isolated from the cells contained approximately the same specific activity as the added methionine. It was concluded that synthesis of methionine was completely inhibited by 2-chloro-4-aminobenzoic acid under these conditions (48). An appreciable incorporation of the carbon of the methyl group into the fatty acids of the organism occurs under these conditions (49).

p-Hydroxybenzoic acid and precursors of aromatic compounds.—Growth of a mutant strain of *E. coli* requiring shikimic acid or dehydroshikimic acid is stimulated by a mixture of tyrosine, phenylalanine, tryptophan, PAB, and *p*-hydroxybenzoic acid. PAB in moderate concentrations serves as a precursor of *p*-hydroxybenzoic acid, but at higher concentrations inhibits competitively

at an index of about 100 the utilization of *p*-hydroxybenzoic acid. The requirement for *p*-hydroxybenzoic acid can also be replaced by a mixture of methionine and lysine. Mixtures of intermediates such as cystathionine and diaminopimelic acid are also effective. *p*-Nitrobenzoic acid competitively inhibits the utilization of both PAB and *p*-hydroxybenzoic acid. 4,4′-Dihydroxydiphenyl sulfone behaves as a competitive antimetabolite of *p*-hydroxybenzoic acid, and aspartic acid potentiates the actions of competitive inhibitors of *p*-hydroxybenzoic acid (50). *p*-Hydroxybenzoic acid reverses the inhibitory effect of PAB on rickettsiae under certain conditions (51).

β-Indolylacetic acid has been reported to have a competitive action against sulfonamides for *Staphylococcus aureus* (51a) which suggests the possibility that the indole derivative may serve as a precursor of PAB in this organism.

Folic acid and related factors.—A synthetic factor, 5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid-SF; leucovorin), has been found to have biological activity similar to natural folinic acid in promoting the growth of *L. citrovorum* 8081 and reversing the toxicity of x-methyl folic acid for *L. casei* and *S. faecalis R* (29 to 33). Folinic acid also reverses more effectively than folic acid the toxicity of 4-amino-4-desoxyfolic acid (aminopterin) for *L. citrovorum* 8081 (52), mice (53, 54), rats (55), and pernicious anemia patients (56), and chicks in preventing the stilbesterol stimulation of the oviduct (57). Similarly, folinic acid is more effective than folic acid in preventing the toxicity of 4-amino-4-desoxy-N^{10}-methylfolic acid (A-methopterin) in mice (58), and the increase in survival time of leukemic (A_K4 and A_K4R strains) mice treated with 9-methylfolic acid (59).

Hydrogenation of 4-amino-4-desoxyfolic acid and its formylated derivative decreases the inhibitory activity of the analogue for *L. citrovorum*, but hydrogenation of the formylated derivative of 4-amino-4-desoxyfolic increases the inhibitory activity for *S. faecalis* (59a).

Tetrahydrofolic acid is 1/3 and 1/40 as effective as the synthetic folinic acid in reversing the toxicity of 4-amino-4-desoxyfolic acid for mice and in promoting growth of *L. citrovorum* 8081, respectively (60).

The acid degradation product of a synthetic folinic acid which is an imidazolinium salt (32) is 1/10 as active as the original compound in reversing the toxicity of 4-amino-4-desoxyfolic acid for mice, but is equally active in promoting the growth of *S. faecalis R*. It is less active than folinic acid in treatment of pernicious anemia patients (61).

Crystalline natural folinic acid isolated from liver is approximately twice as active as the synthetic product (folinic acid-SF, leucovorin), but has the same ultraviolet absorption spectrum and undergoes the same degradation in acid with the formation of an imidazolinium salt. The creation of a new asymmetric center in the synthesis of the tetrahydro derivative and the loss of such a center on acid degradation and oxidation could explain differences in activities of the acid degradation products of the synthetic and natural factors if it is assumed that the synthetic product is a mixture of two forms, only one of which is active (62, 63, 64). However, the possibility of a rather small structural difference cannot be excluded.

Folic acid affords some protection against the toxicity of 4-amino-4-desoxyfolic acid (aminopterin) for dogs (65), guinea pigs (65), rabbits (65), and tumors in mice (66, 67). Thymine or PAB also has some effect in preventing the toxicity of the analogue in guinea pigs (68). Vitamin B_{12} has a small but consistent effect in reducing the efficacy of 4-amino-4-desoxyfolic acid and its N^{10}-methyl derivative (A-methopterin) in treatment of leukemia in mice (69).

The selection of 4-amino-4-desoxy-N^{10}-methylfolic acid resistant leukemic cells of acute lymphoid leukemia in mice involves a transformation to resistance and dependence, and the possibility that the analogue is used per se in lieu of folic acid is suggested (70). The effects of a number of folic acid antagonists as well as 2,6-diaminopurine on neoplastic disease have been studied in human patients and beneficial effects have been noted in some cases (71).

The effects of dietary 4-amino-4-desoxyfolic acid upon the aerobic and anaerobic metabolism of choline, betaine aldehyde, and betaine indicate that oxidation of choline and betaine aldehyde, anaerobic conversion of betaine aldehyde to betaine, and both aerobic and anaerobic transmethylation to homocysteine are decreased significantly (72, 73, 74). Formate incorporation into nucleic acid is decreased by administration of 4-amino-4-desoxyfolic acid to rats (76, 77), and this amino analogue of folic acid also affects phosphate uptake in endocrine tissues of rats receiving the analogue (75).

The development of fertilized frog eggs (*Rana pipiens*) is inhibited by 4-amino-4-desoxyfolic acid, but increasing concentrations produces less inhibition of growth (78). Typical progestational reactions in the uterus of laboratory animals as induced by progesterone can be inhibited by simultaneous administration of 4-amino-4-desoxyfolic acid (79).

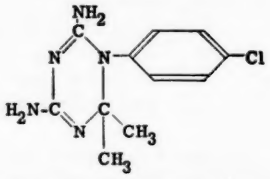
Similar to dehydroisoandrosterone acetate (80), relatively high concentrations of cortisone acetate support the growth of *S. facealis R* and *L. citrovorum* 8081 in place of folic acid and folinic acid, respectively. The toxicity of 4-amino-4-desoxyfolic acid is also reversed by cortisone acetate for *L. citrovorum* but not for mice in which cortisone increased the toxicity of the inhibitor (81, 82).

Albino rats receiving succinylsulfathiazole in a purified diet excreted approximately 5 to 10 times as much urinary glutamic acid as controls. Folic acid reversed the effect of the succinylsulfathiazole (83). The glutamic acid excreted appears in the urine in the form of a heat and alkali-labile compound (84).

4-Amino-4-desoxyfolic acid (aminopterin) and N^{10}-methylfolic acid which inhibit the growth of many organisms replace folic acid in promoting the growth of *Tetrahymena geleii W* and are 17 and 2 per cent, respectively, as active as the vitamin. Pteroylaspartic acid and 4-amino-4-desoxypteroylaspartic acid decrease but do not replace the folic acid requirement of the organism. Folic acid conjugates and folinic acid are all approximately equally effective in promoting the growth of the organism. The 9- and 10-methyl and

9,10-dimethyl derivatives of 4-amino-4-desoxyfolic acid are inhibitory. The last compound is competitive; the inhibition index at half-maximum growth is approximately 1000, but the ratio obtained with the first two compounds decreases markedly with increasing concentrations. It is suggested that destruction of the inhibitor occurs at low concentrations (85).

A new type of inhibitory compound related to folic acid and its derivatives is represented by 4,6-diamino-1-(*p*-chlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine:



This compound inhibits *S. faecalis* 8043 and *Lactobacillus arabinosus* 17-5 at very low concentrations. The inhibition is prevented by the folinic acid group, dihydrofolic acid, N^{10}-formylfolic acid, and thymine but not by folic acid. Organisms such as *L. citrovorum* requiring the folinic acid group are not affected by the inhibitor except at very high concentrations. The inhibitor is six times as effective as quinine against *Plasmodium lophurae* in ducks (86).

Of a group of 3′,5′-dihalopteroyl derivatives, 4-amino-4-desoxy-10-methyl-3′,5′-dichloropteroyl glutamic acid, and the corresponding 9,10-dimethyl derivative are about three times as effective as 10-methylpteroylglutamic acid as an inhibitor of *S. faecalis R.* 3′,5′-Dichloro- and 3′,5′-dibromopteroylglutamic acid are approximately 0.61 and 0.39 per cent as effective as folic acid in promoting growth of the organism. Other compounds such as 3′,5′-dichloro and 3′,5′-dibromo derivatives of 4-amino-4-desoxypteroylglutamic acid, 3′,5′-dichloro-10-nitroso-4-amino-4-desoxypteroylglutamic acid, and 3′,5′-dichloropteroylaspartic acid have some inhibitory activity, but less than that of 10-methylpteroylglutamic acid (87).

2-Alkylamino analogues of folic acid similar to the 4-alkylamino derivatives have less antagonistic action against *S. faecalis R* than 4-amino-4-desoxypteroylglutamic acid (88, 89), and 4-alkoxyl derivatives of folic acid are too labile to isolate, folic acid being produced upon hydrolysis (90). 2-Hydroxy-2-desaminofolic acid possesses 0.02 per cent of the activity of folic acid for *S. faecalis* (91).

Of a group of 34 pterins, all 2,4-diamino- and 2-amino-4-alkylamino, a number of 2-amino-4-hydroxy and one 2-aminopteridine were reported to be inhibitory to *L. casei* in a folic acid medium. Some, such as 2,4-diamino-6,7-dimethylpteridine, 2,4-diamino-6,7-di-*p*-chlorophenylpteridine and 2-amino-4-dimethylamino-6,7-dimethylpteridine, are potent inhibitors even in the presence of purines and thymine. The inhibitions resulting are in some cases

partially reversed by higher concentrations of folic acid. Adenine reverses noncompetitively the toxicity of xanthopterin, 2,4-diamino-6-hydroxy- and 2,4-diamino-6,7-dihydroxypteridine. Adenine and thymine combined similarly reverse the toxicity of leucopterin, 2-amino-4-hydroxy-6,7-dimethylpteridine, and 2-amino-4-di(β-hydroxyethyl)amino-6,7-dimethylpterine. Some of the 2-amino-4-hydroxypteridines become potent inhibitors in the presence of *p*-aminobenzoylglutamic acid even though some are inactive in the absence of the peptide (92, 93).

The bacteriostatic and vibriostatic activities of 6,7-diethyl-, 6,7-diisopropyl-, and 6,7-diphenyl- derivatives of 2,4-diaminopteridine, 2,4-diamino-10-methyl-10H-indolo(3,2-g)pteridine, and 4-amino-4-desoxyfolic acid (aminopterin) have been compared with *S. faecalis* and *Vibrio comma*. For the latter organism, sulfaguanidine inhibition but not inhibition by the pteridines is prevented by PAB (94). 2,4-Diamino-6,7-diisopropylpteridine and 2,4-diamino-10-methyl-10-H-indolo(3,2-g)-pteridine afford some protection for mice infected with *V. comma* (95).

In many cases, it is difficult to determine whether a particular inhibitor somewhat structurally related to a metabolite is antagonistic specifically to the metabolite. The value of cross-resistance and synergism in determining the site of action in such cases is illustrated by the evidence that a chloroguanide-resistant strain of *P. gallinaceum* was cross-resistant to 2,4-diamino-6,7-diphenylpteridine but not to 2,4-diamino-5-(*p*-chlorophenoxy)-6-methylpyrimidine. Also chloroguanide (Paludrine) and the last compound are not synergistic (96). The toxicities of all three compounds are potentiated by sulfonamides and reversed by folic acid (97, 98). Similar results on the effect of sulfonamides and folic acid on the toxicity of these compounds have been obtained previously with lactobacilli (99, 100).

Vitamin B_{12}.—Associated closely with the functioning of PAB, vitamin B_{12} increases the sulfanilamide inhibition indices for the various functions of PAB threefold, indicating a role of the vitamin in the incorporation of single carbon units in methionine, purines, serine, and thymine (5).

While no inhibitor competitively reversed by vitamin B_{12} has been reported, several analogues of possible precursors of the 5,6-dimethylbenzimidazole moiety have been found to exert inhibitory effects related to vitamin B_{12} and possibly riboflavin, which possesses a similar aromatic moiety.

The toxicity of 1,2-dichloro-4,5-diaminobenzene for *S. aureus* is prevented in a competitive manner over a limited range of concentrations by 1,2-dimethyl-4,5-diaminobenzene and to a lesser extent by *o*-phenylenediamine. Riboflavin and vitamin B_{12} combined do not affect the inhibition, but some slight effect is noted with *L. casei* and *Streptococcus hemolyticus* H-96-D. The analogue is ineffective against mice (101). The dichloro compound caused a decrease in production of both riboflavin and vitamin B_{12} by *Bacillus megatherium*, while 1,2-dimethyl-4,5-diaminobenzene stimulates production of vitamin B_{12} (102).

Of some 35 structural analogues of 1,2-dimethyl-4,5-diaminobenzene, nine

were found to be sufficiently toxic for *S. aureus* to warrant further investigation. The inhibitory effect of two of these compounds, 1,2-dichloro-4,5-diaminobenzene and 1,2-dimethyl-4-amino-5-hydroxybenzene, was reversed by 1,2-dimethyl-4,5-diaminobenzene in a competitive manner over about a six-fold range of inhibitor concentrations, and the toxicities of four compounds, 1-methyl-2-chloro-4-amino-5-hydroxybenzene, 1,2-dichloro-4-amino-5-hydroxybenzene, 1,2-dichloro-4-amino-5-nitrobenzene and 1-methyl-2-chloro-4-nitro-5-hydroxybenzene, were decreased slightly by 1,2-dimethyl-4,5-diaminobenzene. In *B. megatherium*, not only 1,2-dichloro-4,5-diaminobenzene and 1,2-dimethyl-4-amino-5-hydroxybenzene but also 1-methyl-2-chloro-4-amino-5-hydroxybenzene and 1-methyl-2-chloro-4-nitro-5-hydroxybenzene at concentrations which do not reduce growth of the organism cause a marked reduction in the amount of vitamin B_{12} synthesized by the organism (103).

The toxicity of 1,2-dimethyl-4,5-diaminobenzene over a narrow range of concentrations is competitively antagonized by vitamin B_{12} in *Lactobacillus lactis;* however, such is not the case with inhibitions by 5,6-dimethylbenzimidazole and its analogues. With suboptimal concentrations of vitamin B_{12}, several substituted anilines support optimal growth of the organism but do so by a mechanism other than stimulation of vitamin B_{12} synthesis (104).

Oxidation of vitamin B_{12} with hydrogen peroxide in acidic solution results in the formation of a reaction mixture which inhibits the growth of *Lactobacillus leichmanii* 4797. In the presence of high concentrations of vitamin B_{12}, approximately twice as much of the reaction mixture is required for inhibition of growth (105).

α-Ribazole (1-α-D-ribofuranosido-5,6-dimethylbenzimidazole), a degradation product of vitamin B_{12}, elicits a marked vitamin B_{12}-like response when fed to rats, is about 0.25 per cent as active as vitamin B_{12}, and is 20 to 50 times as active as 5,6-dimethylbenzimidazole and 1,2-diamino-4,5-dimethylbenzene (105a, 106, 107). β-Ribazole also gives significant responses. These compounds do not replace vitamin B_{12} for lactobacilli requiring vitamin B_{12}, but pseudo-vitamin B_{12} which contains adenine in place of the benzimidazole moiety of vitamin B_{12} is active for such lactobacilli but not for some animals (108).

Biotin.—Biocytin, a naturally occurring biotin conjugate, which is just as active as biotin for *L. casei* but is inactive in replacing biotin for *L. arabinosus*, has been found to be a conjugate of biotin with the ϵ-amino group of lysine (109). A number of biotin derivatives such as biotinamide, N-biotinyl-*p*-aminobenzoic acid, and N-biotinyl-β-alanine have a spectrum of microbiological activity similar to that of biocytin (110).

The utilization of oxybiotin (O-heterobiotin), the oxygen analogue of biotin, occurs without conversion to biotin in many organisms. Another analogue, γ-(3,4-ureylenecyclohexyl)butyric acid, which is a competitive inhibitor of biotin in many organisms, has been found to replace biotin in promoting the growth of *Leuconostoc dextranicum elai*. For this organism,

dl-desthiobiotin and *dl*-oxybiotin are just as active as (+)-biotin. *Leuconostoc mesenteroides* 683 and 9135 and *L. dextranicum* 8086 similarly do not show configurational specificity (111).

4-(Imidazolidone-2)-caproic acid, homobiotin, norbiotin, and hexahydro-2-oxo-4-hydroxybutylfuro-3,4-imidazole are inhibitory for H-37-Rv tubercle bacilli. Both biotin and oxybiotin prevent the inhibitions. Desthiobiotin also prevents the toxicity of the first analogue (112).

Biotinol, the alcohol analogue of biotin, is as effective as biotin in curing egg-white injury in rats and in increasing the excretion of biotin in the urine of human beings. The analogue is inactive in replacing biotin for microorganisms such as *L. casei*, *L. arabinosus* and *Saccharomyces cerevisiae* (113).

The reaction between avidin and biotin has been studied with radioactive biotin and found to be practically complete; however, measurable dissociation does occur and on the basis of equilibrium measurements an expression of the type $K=(A)\ (B)^2/(AB_2)$ describes the system over a considerable range of concentrations. An average value for K of 0.6×10^{-21} under the experimental conditions was obtained (114). This slight dissociation of the biotin-avidin complex may account for the inability of avidin to prevent the growth of microorganisms in the presence of oleic acid which reduces the requirement for biotin to an extremely low amount.

The nicotinic acid group.—High concentrations of a vitamin can actually inhibit the functions of the coenzyme derived from the vitamin. Thus, high concentrations of nicotinamide lower the endogenous respiration of rat liver homogenates; and in a purified malic dehydrogenase system, nicotinamide competitively inhibited diphosphopyridine nucleotide which was found to have 3100 times the affinity of nicotinamide for the apoenzyme (115).

While pyridine-3-sulfonic acid has no effect, 3-acetylpyridine fed to dogs greatly increased the excretion of N^1-methylnicotinamide, but produced no deleterious effects. In a nicotinic acid deficient dog, this effect of 3-acetylpyridine was accompanied by manifestations of blacktongue (116). Nicotinic acid or tryptophan (117) afford partial protection for rats against the toxicity of borrelidine, a crystalline antibiotic from the culture broth of a species of *Streptomyces* (118). Desaminodiphosphopyridine nucleotide previously reported to have activity in replacing diphosphopyridine nucleotide in yeast alcohol dehydrogenase (119) has been found to be equally active in some enzyme systems, but is considerably less active than the coenzyme for many enzyme systems (120).

Pantothenic acid.—The biological role of the *Lactobacillus bulgaricus* factor(s), known to contain bound pantothenic acid (121, 122), in the biosynthesis of coenzyme A was investigated using analogues of pantothenic acid (123). Pantoyltaurine competitively inhibited pantothenic acid for certain lactic acid bacteria, while concentrates of the factor(s) gave essentially noncompetitive reversal. The failure of conjugated pantothenic analogues, such as pantoyltauramido-3,5-dibromobenzene to be completely reversed by the factor(s) may result from inhibitions at a later stage in the

biosynthesis. Pantoyltauramido-4-anisole has been reported to be an inhibitor of the *L. bulgaricus* factor(s) (124).

A synthetic *L. bulgaricus* factor, N-pantothenyl-β-mercaptoethylamine (pantetheine) (125) is transformed into coenzyme A by a crude pigeon liver preparation used in the acetylation assay of coenzyme A (126). However, pantetheine is not utilized by several organisms which require pantothenic acid for growth.

The competitive inhibition of the utilization of pantothenic acid by ω-methylpantothenic acid, previously reported for certain bacteria, protozoa, rats, and chicks, has been extended to mice for which the "interference ratio," the ratio of inhibitor to metabolite at which survival time is not increased compared to vitamin-deficient controls, was found to be approximately 100 (127).

Pantonyl-β-alanine, an analogue of pantothenic acid in which one hydroxyl is replaced by an amino group, is utilized by *E. coli* probably by splitting of the molecule into its component parts (128).

The synthesis of pantothenic acid-2′ and 4′-phosphates has received attention as possible degradation products of coenzyme A (129, 130). The phosphates are inactive for both *Acetobacter suboxydans* and *L. arabinosus* and have not as yet been identified as degradation products of coenzyme A.

On the basis of the ability of pantothenic acid to prevent the toxicity of higher concentrations of streptomycin than are required for inhibition of growth of *Saccharomyces fragilis* (ATCC. No. 2360) in the presence of β-alanine, it is suggested that the antibiotic may prevent pantothenic acid synthesis from β-alanine (131).

The vitamin B_6 group.—2-Methyl-3-amino-4,5-bishydroxymethylpyridine is a reversible antagonist for vitamin B_6 in a mutant strain of *Neurospora sitophilia* (132). Desoxypyridoxine fed to human subjects does not change the level of N^1-methylnicotinamide excreted (133) but induces an 8 to 10-fold increase in xanthurenic acid excretion in urine after the administration of tryptophan (134). However, desoxypyridoxine is reported to inhibit the natural increase in nicotinic acid as well as ascorbic acid in germinating pulses (*Phaseolus mungo*, *Vigna sinensis*, and *Cajanus cajan*). The inhibition is reversed by pyridoxine (135). Certain pyridoxylamines, prepared by hydrogenation of pyridoxylideneamines formed from pyridoxal and various amines, are all highly active in replacing pyridoxine for rats (136).

Riboflavin.—Yeast flavokinase phosphorylates dichloroflavin [6,7-dichloro-9-(D-1′-ribityl)-isoalloxazine] at a rate slightly greater than that of riboflavin and phosphorylates arabitylflavin at a considerably slower rate. Isoriboflavin, galactoflavin, dulcitylflavin, and sorbitylflavin are not phosphorylated and do not inhibit the phosphorylation of riboflavin. Lumiflavin on the other hand does inhibit phosphorylation of riboflavin. Isoriboflavin, dichloroflavin, and glactoflavin inhibit cytochrome-*c* reductase (triphosphopyridine nucleotide) from yeast, D-amino acid oxidase from kidney,

and L-amino acid oxidase from snake venom, but the inhibitions are not extensive enough to account for the inhibitory effects of the compounds on various organisms (137).

Since mammary carcinomas in C3H mice decreased during severe riboflavin deficiency (138) and lymphosarcoma (6C3H-ED) is also retarded in mice deficient in riboflavin or treated with riboflavin antagonists (139, 140), diethylriboflavin was administered to rats to obtain a 33 per cent inhibition of growth of Walker Carcinoma 256 (141).

6,7-Diethyl-9-(D-1′-ribityl)-isoalloxazine replaces riboflavin in promoting growth of *L. casei*. At limiting concentrations over a 72 hr. growth period, the analogue and riboflavin produce indistinguishable growth stimulations; however, if the incubation period is extended to 120 hr., riboflavin is superior to the analogue in permitting acid formation (142). Similar results are obtained with *Bacillus lactis acidi*, but the analogue does not appear to have growth promoting activity for rats (143).

With supplements of either natural products, e.g., fish meal, or L-lyxoflavin (150 μg. per day) which is devoid of riboflavin activity for rats and *L. casei*, weight gains of 20 to 35 per cent above control groups have been observed with rats maintained on a diet free of animal protein and containing thyroid powder and vitamin B_{12} supplements (144). This riboflavin analogue which competitively inhibits the utilization of riboflavin for several microorganisms such as *L. casei* was previously reported to have been isolated from human heart (145). The possibility that L-lyxoflavin may represent a new vitamin group has been suggested, but more experimental evidence concerning this effect on animals will be required in order to determine the mode of action of this compound in increasing the rate of growth of rats.

Thiamine.—Neopyrithiamine, a compound which has the structure originally assigned to pyrithiamine but which is several times as active as pyrithiamine (146, 147, 148), promotes the growth of *Saccharomyces carlsbergensis* 4228 in a medium used for assay of vitamin B_6. The organism grows well in the absence of both vitamin B_6 and thiamine, but supplements of thiamine prevent growth of the organism. The inhibition is overcome by supplements of vitamin B_6 or a thiamine antagonist, neopyrithiamine (149).

Neopyrithiamine inhibits production of cocarboxylase in the blood of normal chickens and in cell-free preparations. Pyrithiamine pyrophosphate but not pyrithiamine inhibits the combination of cocarboxylase with yeast apocarboxylase. Since blood destroys both the analogue and thiamine pyrophosphates, it is not possible to determine whether neopyrithiamine prevents the formation of cocarboxylase, or the pyrophosphate is formed and prevents the combination of cocarboxylase with the apoenzyme. It is suggested that some additional function of thiamine may be the primary point at which the inhibitor exerts its effect since amounts larger than those toxic to the animal are necessary to prevent the cocarboxylase formation (150).

Microsporum audouini is inhibited *in vitro* by neopyrithiamine, and thiamine reversed the toxicity of the inhibitor (151). Oxythiamine triphos-

phate is an effective inhibitor of the formation of carbon dioxide and acetaldehyde by the carboxylase system of washed yeast supplemented with either thiamine pyro- or triphosphate and manganese. If the coenzyme is added before the inhibitor, the effectiveness of the inhibitor is less marked (152). Oxythiamine, though a thiamine antagonist *in vivo* for certain organisms, is ineffective in this system.

Homothiamine-glycol is a competitive antagonist of thiamine but is only 1/16 as effective as neopyrithiamine against *Lactobacillus fermenti* and considerably less effective against root meristem of the pea (153).

Reversion of the stimulatory effect of thiamine on yeast fermentation as a result of growth in the presence of cocaine has been noted. In the presence of cocaine the fermentation is further depressed by thiamine (154). Such results may have possible implications in regard to drug addiction.

Pyrimidines and Purines

Pyrimidines.—The biological effects of analogues of pyrimidines are rather complicated and involved, but the following equations indicating a probable mechanism of biosynthesis of nucleic acid pyrimidines allow a classification of the biological effects (Fig. 1).

In these equations oxaloacetic and ureidosuccinic acid are pictured as precursors of an intermediate which forms a conjugated form of orotic acid. Oxaloacetic acid and related compounds have some ability to replace pyrimidines in a pyrimidineless strain of *Neurospora* (155, 156) while ureidosuccinic acid can replace orotic acid in supporting growth of *L. bulgaricus* 09 (157).

Certain pyrimidineless strains of *Neurospora* can utilize orotic acid while other strains which accumulate the compound do not utilize it. However, a pyrimidineless mutant which neither utilizes nor accumulates orotic acid has been crossed with a strain which accumulates orotic acid, but the resulting double mutant does not produce orotic acid. In order to account for this data, the cyclic interrelationship of the intermediate X and orotic acid and its derivative as indicated in Figure 1 has been proposed. The mutant which neither utilizes nor accumulates orotic acid has lost the ability to convert X to the orotic acid derivative conjugated with an R_1 group. This conjugated orotic acid is pictured as being converted into conjugated forms of uracil and cytosine which are the precursors of ribonucleic acids. Uracil, uridine, and cytidine are represented as products of side reactions, and the ability of organisms to utilize the compounds depends upon their ability to convert them to the normal conjugated intermediates. Some form of these pyrimidines has a role in the biosynthesis of the thymine component of desoxyribonucleic acid through a conjugated intermediate, possibly closely related to thymidine. This interconversion involves the incorporation of a single carbon unit at one stage and at least the ureido part of the pyrimidines. Thymine is indicated not to be the normal intermediate but the product of a side reaction, and again the ability of an organism to use such a product

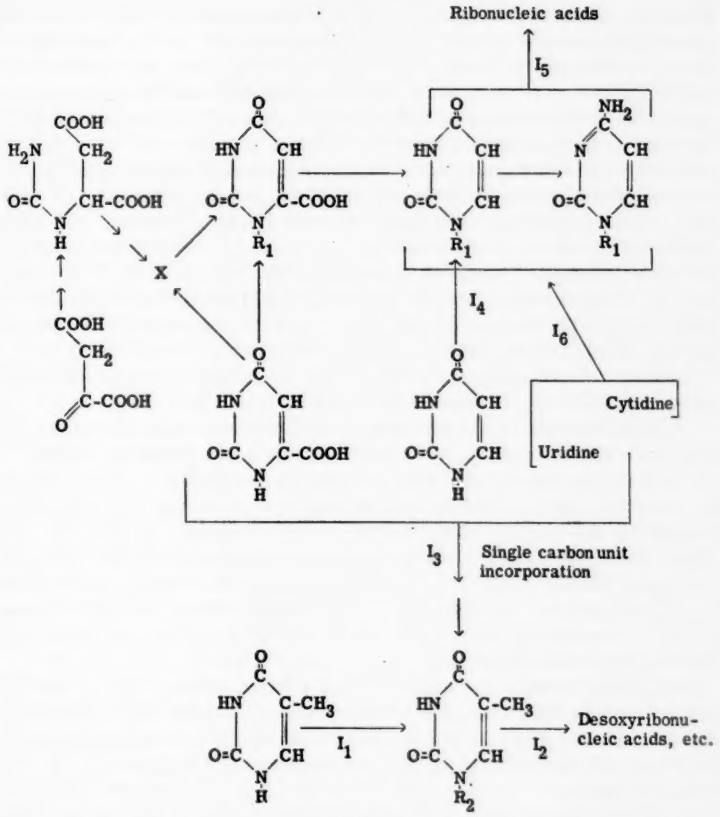


FIG. 1.

depends upon its ability to convert the compound to a conjugated form. Even in such a simplified scheme there are obviously many points at which a pyrimidine analogue might competitively inhibit the utilization of a normal intermediate. For example, in *L. casei* either 2-thio-thymine-(5-methyl-2-thiouracil) (158) or 5-bromouracil (159–161) inhibits in a competitive manner growth stimulated by thymine in the absence of folic acid but has no effect on growth stimulated by folic acid. These inhibitors appear to act at the point indicated by I_1, and become ineffective when the normal synthesis is established by supplements of folic acid which is involved in the single carbon unit incorporation in the biosynthesis of the conjugate of thymine.

Further evidence for such a mechanism is the fact that thymine com-

petitively prevents the incorporation of 5-bromouracil-Br^{82} into *S. faecalis*, presumably into the nucleic acid fractions, while folic acid in reversing this inhibitor functions without displacing 5-aminouracil in the same manner (162). On the other hand, 5-bromouracil (159, 160, 161) may prevent the utilization of the conjugate of thymine as indicated by I_2 so that it exerts its effect in the presence of either folic acid or thymine; and increasing the concentration of either may affect the concentration of the conjugate so that reversal of the inhibition can be obtained by increasing either over a limited range of concentrations. 5-Nitrouracil has still a different effect in that uracil competitively prevents the inhibition, but thymine prevents the inhibition in a noncompetitive manner of a product (159, 160, 161, 163). Thus, it appears that this inhibitor prevents the formation of the thymine conjugate as indicated by I_3. Pyrimidine analogues, such as thiouracil (158) and 5-hydroxyuracil (159, 160, 161), the toxicity of which is reversed by uracil for *L. casei* but is not affected by thymine or folic acid, apparently interfere with other functions of uracil as indicated by I_4 and I_5.

5-Aminouridine, 5-hydroxyuridine, 3-methyluridine, 5-bromouridine, and 5-chlorouridine competitively inhibit the utilization of either uridine or cytidine by *Neurospora* 1298, which requires pyrimidines for growth. Uridine is about five times as effective as cytidine in preventing the inhibitions. Growth of the mutant stimulated by uracil or growth of the wild type *Neurospora* is prevented by 5-aminouridine and 5-hydroxyuridine but not by 3-methyluridine or 5-chlorouridine. It is suggested that uridine and cytidine are not normal intermediates in the biosynthesis of the pyrimidine components of nucleic acid and that uracil is utilized by a route not involving uridine and cytidine (164, 165).

Thiouracil inhibits the biosynthesis of tobacco mosaic virus in isolated sterile disks of tobacco leaf tissue under continuous illumination and supply of nutrients. The inhibitor is "virostatic" and affects only multiplication of the virus. The inhibition is partially reversed by the simultaneous presence of uracil (166).

5-Chlorouridine inhibits the propagation of Theiler's GD VII virus and uptake of P^{32}-phosphate by the ribonucleic acid fraction of minced, one day old mouse brain. Uridine partially reverses the inhibitions (168).

Diazobarbituric anhydride [5,7-dihydroxy-(1,2,3)-oxadiazolo-(5,4-*d*)-pyrimidine] inhibits the assimilation of cytosine and 5-methylcytosine as nitrogen sources for *Torula utilis* and *S. cerevisiae* Hansen, probably by inhibition of cytosine deaminase (169).

In the absence of uracil supplements, *L. casei* is inhibited by 4-propyl-2-thiouracil, 5-benzyl-2-thiouracil, and 5-(3,4-dimethylbenzyl)-2-thiouracil and less effectively by 5-propyl- and 5-methyl-2-thiouracil. In all cases, the activities were less than that of thiouracil, and the inhibitions were overcome by uracil (167).

Purines.—Antimetabolites such as sulfonamides which allow the accumulation of 5-amino-4-imidazolecarboxamide in certain organisms under

conditions of limiting purine biosynthesis have given some insight into the mechanism of purine biosynthesis. Aminomethane sulfonic acid, the sulfonic acid analogue of glycine, has been shown to inhibit the multiplication of P_1 bacteriophage of *E. coli*. While the inhibition is prevented by xanthine, it was not shown that the analogue actually inhibited glycine utilization (170). While a number of purine antagonists have been prepared, these inhibitors have not been utilized appreciably in the study of biochemical interrelationships. Two of the more generally effective purine inhibitors, 2,4-diaminopurine and 8-azaguanine, have been studied extensively and particularly for their effect upon malignant growth. The former compound is in general an antagonist of adenine (171), and the latter inhibitor is in general an antagonist of guanine or a related derivative. The synthesis of nucleic acids in mice as measured by incorporation of C^{14} from bicarbonate into nucleic acid purines is inhibited by both of these purine antagonists (172). Studies on the toxicity of 2,4-diaminopurine have been extended to cultures of embryonic and sarcomatous rodent tissues (173), and further evidence for the mode of action of 8-azaguanine in tumor inhibition has been presented (67, 174, 175). Guanine incompletely reverses the toxicity of the inhibitor on adenocarcinoma 755. Guanylic acid gives a progressive reversal of the tumor-inhibitory action of the guanine analogue as contrasted with the minimal and nonprogressive reversal obtained with guanine (176). The concentration of a deaminating enzyme, which converts 8-azaguanine to the noncarcinostatic 8-azaxanthine, is low in 8-azaguanine-susceptible tumor as compared with a nonsusceptible tumor and several normal tissues (177, 178).

Reduced concentrations of essential metabolites decrease the effectiveness of 8-azaguanine in inhibiting *Tetrahymena*. It is proposed that the restriction of metabolites reduces 8-azaguanine incorporation and thereby reduces the toxicity of the inhibitor (179). 2,6-Diaminopurine inhibits the utilization of hypoxanthine and less effectively adenine but does not inhibit the utilization of adenosine or inosine by adenineless strains of *Neurospora*. Guanine inhibits the utilization of hypoxanthine while guanosine prevents the utilization of inosine as well (180).

2,6-Diaminopurine inhibits the multiplication of Russian Spring-Summer Encephalitis virus in tumor tissue media and increases the number of survivors of mice infected with the virus (181, 182).

In a study of the effects of 100 purines on *L. casei* in a variety of media, it was found that structural changes, such as the introduction of an 8-hydroxyl or 8-chloro group, methylation of the imidazole nucleus, and alkylation of the amino group of the aminopurines, result in a diminution of the growth promoting or inhibitory activity of the parent compound. Replacement of hydroxyl groups of guanine and hypoxanthine by a mercapto group results in inhibitory substances, the effects of which are reversed by purines. 8-Arylpurines are moderate to strong inhibitors, but do not clearly fall into the competitive inhibitory class since reversal by adenine or folic acid is

slight; 7- and 8-methyldiaminopurines are reversed by folic acid, but 7-methyldiaminopurine toxicity is not reversed by adenine or other purines. 9-Methyldiaminopurine is reversed by any purine but not by folic acid (93, 183).

While 6-hydroxyimidazo-1,2,3-triazine (2-azahypoxanthine) has very slight hypoxanthine activity for *Lactobacillus brevis*, 6-aminoimidazo-1,2,3-triazine (2-azaadenine) is toxic to several organisms including bacteria, yeast, and mice. Adenine reverses the toxicity noncompetitively for *L. brevis* and *S. cerevisiae* while hypoxanthine exerts some slight effect. Hypoxanthine and adenine prevent the toxicity of the inhibition somewhat competitively for *L. arabinosus* and mice (184).

2-Azaadenine (6-aminoimidazo-1,2,3-triazine) and 2-azahypoxanthine serve as substrates for milk xanthine oxidase. Azaadenine is oxidized at a rate somewhat faster than hypoxanthine and is converted to 8-hydroxy-azaadenine. The oxidation of hypoxanthine and xanthine was prolonged 2- to 4-fold in the presence of equimolar amounts of azaadenine. Not only was the inhibition due to the oxidation of the analogues, but the product, 8-hydroxy-azaadenine, exerted an inhibitory effect (185).

Amino Acids

Aromatic amino acids.—Of the amino acid analogues, those related structurally to phenylalanine have received considerably more attention than analogues of other amino acids. This probably resulted from the ease of synthesis of a number of these compounds and the unusual frequency of inhibitory activity among these analogues. Alteration of the side chain, modification of the phenyl nucleus through the use of isosteric ring structures, and substitution of the phenyl nucleus of phenylalanine have all been successful in producing inhibitory analogues. These include β-2- and β-3-thienylalanine (186 to 193), β-hydroxyphenylalanine (188), β-2- and β-3-furylalanine (194, 195), β-2-pyrrolealanine (196), 4-pyridinealanine (197), and several halogenated phenylalanines (198).

Following studies on acute toxicity of 3-fluorophenylalanine and 3-fluorotyrosine (199) and the demonstration of competitive inhibition by these analogues of the utilization of the corresponding amino acid by *Neurospora* (200), there has been considerable interest in these and the other isomeric fluoro derivatives of these amino acids. Thus, the toxicity of 4-fluorophenylalanine for *Pseudomonas aeruginosa* ATCC 9027 (201), *L. arabinosus* (202), and *S. cerevisiae* (203) and to some extent for rats (204, 205) is prevented by phenylalanine but not by tyrosine. The inability of phenylalanine to prevent completely the toxicity of the fluoro derivative for rats has been interpreted to result from the production of inorganic fluoride from the organic derivative (204). 2-Fluorophenylalanine is slightly more toxic than the 4-fluoro derivative (205), but studies on the mechanism of the toxicity are still incomplete.

Tyrosine prevents the toxicity of a few analogues of phenylalanine in a

noncompetitive manner suggesting a function of phenylalanine in tyrosine biosynthesis. Tryptophan prevents the toxicity of phenylalanine antagonists for a number of organisms in such a manner as to indicate that the presence of this aromatic amino acid stimulates the production of a higher concentration of phenylalanine by the organism. The inhibitory effect of *p*-hydroxycinnamic acid on the production of tyrosine in *S. cerevisiae* is counteracted by phenylalanine, tryptophan, and nicotinic acid (206).

The growth inhibiting effects on *E. coli* of indoleacrylic acid, coumarin, 2-methyl-1,4-naphthoquinone, and β-hydroxyphenylalanine are antagonized by tryptophan, phenylalanine, and nicotinic acid. The inhibitory effect of indoleacrylic acid is also antagonized by anthranilic acid, indole, serine, kynurenine, hydroxyanthranilic acid, and perhaps ornithine (207).

While the biosynthesis of nicotinic acid from tryptophan and the existence of interrelationships of aromatic amino acids are now well established, the exact mechanisms by which the antagonisms indicated above are exerted are not too well defined.

The growth of phenylalanine-requiring bacteria, *L. mesenteroides* P-60 and a phenylalanine-requiring mutant of *E. coli* (M-83-5), is inhibited by DL-*o*-hydroxyphenylalanine (208). The inhibition is prevented in a somewhat competitive manner by phenylalanine, the ratio of concentrations of inhibitor to phenylalanine increasing about two-fold as the concentrations are varied over a 5 to 10-fold range. Wild type strains of *E. coli* (ATCC 9637, 9723) or a tyrosine requiring mutant (M-83-9) are not affected by this inhibitor even at concentrations as high as 2 mg. per ml., but a wild-type strain of *E. coli* which has been reported to be inhibited by tyrosine (209) at very low concentrations only in the absence of phenylalanine is similarly affected by *o*-hydroxyphenylalanine. The data concerning reversal of increasing concentrations of the inhibitor by increasing concentrations of phenylalanine suggest that this inhibitor at low concentrations probably prevents the biosynthesis of phenylalanine since the amount of phenylalanine necessary for reversal of the toxicity of increasing concentrations of inhibitor increases less than two-fold for a 10-fold increase in concentration of inhibitor. DL-Tryptophan was 1/20 as effective as phenylalanine.

Of a group of 12 new synthetic analogues of phenylalanine, four were reported to inhibit the growth of *E. coli* (210). β-(*p*-Aminophenyl) alanine was inhibitory at low concentrations (0.5 μg. per ml.) while *p*-chloro- and *p*-(7-chloro-4-quinolylamino)-phenylalanine and β,β-diphenylalanine prevented growth of the organism only at higher concentration (1 mg. per ml.). The toxicity of the analogues was prevented by phenylalanine or tyrosine. The same group of analogues does not affect the growth of *L. mesenteroides* P-60 in concentrations up to 100 μg. per ml. (211).

β-(*p*-Aminophenyl)-alanine inhibits the growth of yeast as well as *E. coli*, and the inhibition is reversed by phenylalanine, tyrosine, and tryptophan. *p*-Nitrophenylalanine was less effective but was reversed by a similar group of amino acids (212, 213).

Indoleacrylic acid and β-(3-quinolyl)-acrylic acid inhibit competitively the utilization of phenylalanine by *L. mesenteroides* P-60 at inhibition indices of 69 and 64, respectively. The latter compound exerts a similar effect against *L. arabinosus*. Both *L. arabinosus* and *L. mesenteroides* P-60 are inhibited noncompetitively by β-(2- and 4-)-quinolylacrylic acid. The toxicity of *p*-hydroxycinammic acid for *L. mesenteroides* P-60 is reversed by either phenylalanine or tyrosine (214).

Phenylalanine, tyrosine, and tryptophan reverse the toxicity of low concentrations of chloramphenicol (Chloromycetin) for *E. coli*, but the inhibitory action of higher concentrations of the antibiotic are not affected by these amino acids. Only phenylalanine exerts such an effect for a mutant strain of *E. coli* requiring phenylalanine for growth and for other organisms requiring phenylalanine (215).

Since the reversal by phenylalanine is exhibited only over a very narrow range of concentrations of the inhibitor, chloramphenicol toxicity cannot definitely be ascribed to an antagonism of phenylalanine metabolism. Also, the toxicity is not affected by phenylalanine in some strains of the organism (216). *E. coli* strains resistant to β-2-thienylalanine are rapidly developed while strains resistant to the antibiotic are less resistant than the normal strain to the antimetabolite of phenylalanine (216). The development of resistance to β-2-thienylalanine appears to be the result of biosynthesis of metabolites which reverse the toxicity of the inhibitor (216).

Of two forms of *p*-nitro-β-phenylserine, only one was an antagonist of phenylalanine for *E. coli* (217) and by analogy to the activity of threo-β-phenylserine was assigned the threo configuration. These configurations have been of interest because of their structural relationship to chloramphenicol (218). Mandelic acid is reported to inhibit competitively the conversion of phenyllactic acid to phenylalanine in *L. casei* (219).

Since symptoms of Lansing type poliomyelitis infection in mice are delayed in tryptophan-deficient animals (220), analogues of tryptophan as possible chemotherapeutic agents against this type of infection have been screened. 6-Methyltryptophan has been reported to exert some beneficial effect against the infection in mice (221). 5-Methyltryptophan is converted into 5-methylindole by *E. coli*. The reaction is more rapid in the presence of small amounts of indole (223).

Tryptophan reverses the toxicities of β-(2-benzimidazolyl)-alanine and β-methyl-β-(2-benzimidazolyl)-alanine for *L. mesenteroides* and the toxicities of β-(5-methyl-2-benzimidazolyl)-alanine and β-methyl-β-(5-methyl-2-benzimidazolyl)alanine for *E. coli* (222). The toxicity of D-tryptophan for crown-gall bacteria is partially reversed by xanthurenic acid and 3-hydroxyanthranilic acid (224).

Peptides of aromatic amino acid analogues and natural amino acids were found to be no more effective and in most cases less effective than the free amino acids as growth inhibitors of microorganisms (225).

Methionine.—Modifications of the methionine molecule in the neighborhood of the sulfur atom has in general led to inhibitory analogues of the

amino acid. Thus, ethionine (226, 227), methoxinine (228), norleucine (227), allylglycine (195), crotylglycine (195), and 2-amino-5-heptenoic acid (229), are competitive antagonists of methionine. In *E. coli*, norleucine competitively prevents the functioning of methionine in reactions involving leucine, isoleucine, and valine (230). These four amino acids are often found to be interrelated in competitive inhibitions. In *Achromobacter fischeri*, homocysteine, cystathionine, and homoserine reverse the toxicity of norleucine in a manner suggesting that the conversion of these precursors to methionine is prevented by the analogue. Valine, isoleucine, and leucine as well as thiamine and pantothenic acid also affect the inhibition (231). Transamination reactions involving methionine, valine, isoleucine and leucine appear to be involved in this system.

The effects of methionine sulfoximine, the neurotoxic factor in agenized flour (233, 234), are prevented by methionine in animals (235, 236). Methionine also prevents the decrease in xanthine oxidase activity of the liver resulting from administration of the factor to rats (237). However, for *L. mesenteroides*, the toxicity of the sulfoximine is reversed by glutamine (234, 238).

Methionine or choline prevents the inhibitory effect of ethionine on growth of mice and rats (239, 240), and methionine prevents the development of fatty livers in fasted rats treated with ethionine (241). Complete protection is afforded by methionine given in amounts equal to the analogue (242). L-Ethionine produces a rapid increase in the fatty acid content of the liver of fasted female rats, and of a number of structurally related compounds, only ethione sulfoxide was found active in fasted female rats (242).

Ethionine labeled in the ethyl group and with S^{35} appears to be incorporated into the proteins of rats fed the analogue (243, 244). The labeled ethyl groups remain in a form which can be cleaved by hydriodic acid. Ethionine inhibits choline oxidase of rat liver homogenates, but the inhibition is not reversed by methionine (245).

Administration of methoxinine to rats and mice causes a reduction in food consumption and a weight loss, and the toxic effects are alleviated in mice by administration of methionine. A "pseudolipotropic" effect results from low caloric intake (246, 247).

Methoxinine, ethionine, and methionine sulfoximine inhibit the propagation of the PR8 strain of influenza type-A virus in cells of the chorioallantoic membrane. The inhibition is reversed by L-methionine and partially by homocysteine (248). Similarly, ethionine supresses the biosynthesis of the Lansing strain of poliomyelitis virus grown in a tissue culture medium containing minced human embryonic tissue. The effect is reversed by methionine (249, 250). Some beneficial effects of methionine sulfoximine were obtained in mice infected with the Lansing strain of the virus (251). The propagation of Theiler's virus *in vitro* in a medium containing minced one-day-old mouse brain is inhibited by lysine. The inhibition is partially reversed by methionine (252).

Other amino acids and related metabolites.—Methionine sulfoxide, in

competitively preventing the utilization of glutamic acid in *L. mesenteroides*, appears to prevent the formation of glutamine or a related product since the amide or glutamyl peptides prevent the toxicity of the inhibitor noncompetitively (232). Similar results were previously obtained with *L. arabinosus* (253, 254).

ω-Methyllysine (2,6-diaminoheptanoic acid) inhibits the utilization of lysine in a competitive manner for *S. faecalis R* and *L. mesenteroides* P-60. The analogue in concentrations almost equal to that of lysine completely inhibits growth of the organism (255). Another analogue of lysine, ε-hydroxynorleucine, is utilized by certain mutant strains of *Neurospora* requiring lysine for growth but is inhibitory to other strains (256).

Canavanine, which has been reported to inhibit competitively the utilization of arginine by *E. coli* (257), lactic acid bacteria (257), and *Neurospora* (258), promotes the growth of *Neurospora* 46003R, which also utilizes threonine, isoleucine, α-aminobutyric acid, or homoserine for growth (259). Another mutant, *Neurospora* 51504, utilizes homoserine but not canavanine. The utilization of canavanine is inhibited by arginine or lysine in strain 46003R. Similarly, a strain of *Bacillus subtilis* can utilize either homoserine or canavanine for growth.

For *S. cerevisiae* strain D.C.L. 349, but not for other strains of the organism, uracil is a competitive inhibitor of arginine. The ratio of uracil to arginine at which complete reversal is attained is three as compared to 120 for citruline as a reversing agent. In contrast to the specificity of uracil, canavanine is inhibitory to all strains of the organism, and arginine reverses the toxicity of the analogue (260).

The duration of the lag phase of growth of a leucineless strain of *E. coli* is increased in the presence of increasing concentrations of leucine peptides, but ultimate growth in the presence of equivalent concentrations of leucine and the peptides was identical (261).

L-Amino acid oxidase of cottonmouth venom catalyzed the oxidation of 22 amino acid analogues tested except two with a tertiary α-carboxyl. The amino acid analogues which are inhibitors of certain naturally occurring amino acids were oxidized at rates comparable to that of their corresponding natural amino acid. D-Amino acid oxidase from hog kidney also oxidizes these analogues of natural amino acids, though frequently at a rate much less than for the corresponding natural compound. Also, phenylalanine and β-2- and β-3-thienylalanine were decarboxylated slowly by L-tyrosine decarboxylase of *S. faecalis R*. It is suggested that amino acid antagonists do not inhibit by interfering with enzymatic degradation of amino acids (262).

Malonic acid which competitively inhibits the oxidation of succinic acid inhibits the production of influenza virus *in vitro* by action upon tissue cells (263). Similar results are obtained with fluoroacetate which prevents the oxidation of citrate. These results are considered evidence that the Krebs cycle is essential for the propagation of influenza virus. Fluoroacetate delays development of the Lansing strain of poliomyelitis virus in mice (251).

LITERATURE CITED

1. Woods, D. D., and Fildes, P., *Chemistry & Industry*, **18**, 133–34 (1940)
2. Woods, D. D., *Brit. J. Exptl. Path.*, **21**, 74–90 (1940)
3. Wooley, D. W., *A Study of Antimetabolite* (John Wiley & Sons, Inc., New York, N. Y., 269 pp., 1952)
4. Williams, R. J., Eakin, R. E., Beerstecher, E., Jr., and Shive, W., *The Biochemistry of B-Vitamins* (Am. Chem. Soc. Monograph No. 110, Reinhold Publishing Corp., New York, N. Y., 741 pp., 1950)
5. Shive, W., *Ann. N. Y. Acad. Sci.*, **52**, 1212–34 (1950)
6. Shive, W., and Macow, J., *J. Biol. Chem.*, **162**, 451–62 (1946)
7. Shive, W., and Roberts, E. L., *J. Biol. Chem.*, **162**, 463–71 (1946)
8. Winkler, K. C., and Haan, P. G. de, *Arch. Biochem.*, **18**, 97–107 (1948)
9. Stetten, M. R., and Fox, C. L., Jr., *J. Biol. Chem.* **161**, 333–49 (1945)
10. Shive, W., Ackermann, W. W., Gordon, M., Getzendaner, M. E., and Eakin, R. E., *J. Am. Chem. Soc.*, **69**, 725–26 (1947)
11. Roepke, R. R., Libby, R. L., and Small, M. H., *J. Bact.*, **48**, 401–12 (1944)
12. Ravel, J. M., Eakin, R. E., and Shive, W., *J. Biol. Chem.*, **172**, 67–70 (1948)
13. Winkler, K. C., Haan, P. G. de, and Langerijt, J. van de, *Antonie van Leeuwenhoek J. Microbiol. Serol.*, **15**, 129–35, 136–42 (1949)
14. Snell, E. E., and Mitchell, H. K., *Proc. Natl. Acad. Sci. U. S.*, **27**, 1–7 (1941)
15. Stokstad, E. L. R., *J. Biol. Chem.*, **139**, 475–76 (1941)
16. Stokes, J. L., *J. Bact.*, **48**, 201–9 (1944)
17. Rogers, L. L., and Shive, W., *J. Biol. Chem.*, **172**, 751–58 (1948)
18. Stokstad, E. L. R., Regan, M., Franklin, A. L., and Jukes, T. H., *Federation Proc.*, **7**, 193–94 (1948)
19. Holland, B. R., and Meinke, W. W., *J. Biol. Chem.*, **178**, 7–15 (1949)
20. Woods, D. D., and Lascelles, J., *Nature*, **166**, 649–50 (1950)
21. Bennett, M. A., *Science*, **110**, 589–90 (1949)
22. Stekol, J. H., and Weiss, K., *J. Biol. Chem.*, **186**, 343–50 (1950)
23. Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, **166**, 435–48 (1946)
24. Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, **164**, 485–6 (1946)
25. Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, **170**, 133–46 (1947)
26. Lampen, J. O., and Jones, M. J., and Roepke, R. R., *J. Biol. Chem.*, **180**, 423–34 (1949)
27. Sevag, M. G., Kaft, B. W., and Steers, E., *J. Biol. Chem.*, **185**, 17–25 (1950)
28. Miller, A. K., *Proc. Soc. Exptl. Biol. Med.*, **57**, 151–53 (1944)
29. Shive, W., Bardos, T. J., Bond, T. J., and Rogers, L. L., *J. Am. Chem. Soc.*, **72**, 2817–18 (1950)
30. Flynn, E. H., Bond, T. J., Bardos, T. J., and Shive, W., *J. Am. Chem. Soc.*, **73**, 1979–82 (1951)
31. Pohland, A., Flynn, E. H., Jones, R. G., and Shive, W., *J. Am. Chem. Soc.*, **73**, 3247–52 (1951)
32. May, M., Bardos, T. J., Barger, F. L., Lansford, M., Ravel, J. M., Sutherland, G. L., and Shive, W., *J. Am. Chem. Soc.*, **73**, 3067–75 (1951)
33. Brockman, J. A., Roth, B., Broquist, H. P., Hultquist, M. E., Smith, J. M., Jr., Fahrenbach, M. J., Cosulich, D. B., Parker, R. P., Stokstad, E. L. R., and Jukes, T. H., *J. Am. Chem. Soc.*, **72**, 4325–26 (1950)
34. Woods, D. D., *The Role of Growth Factors in the Synthesis of Amino Acids* (Presented at 120th Meeting of Am. Chem. Soc., New York, N. Y., Sept., 1951)

35. Auhagen, E., *Z. physiol. Chem.*, **283**, 195–201 (1948)
36. Hill, J., *Ann. Trop. Med. Parasitol.*, **44**, 291–97 (1950)
37. Tschesche, R., Kohncke, K. H., and Korte, F., *Chem. Ber.*, **84**, 485–90 (1951)
38. Tschesche, R., Korte, F., and Korte, I., *Z. Naturforsch.*, **6b**, 304–9 (1951)
39. Tschesche, R., Korte, F., and Korte, I., *Z. Naturforsch.*, **5b**, 312–16 (1950)
40. Tschesche, R., and Cremer-Bartels, G., *Z. Naturforsch.*, **5b**, 367–73 (1950)
41. Ware, G. C., *Lancet*, **II**, 865–66 (1951)
42. Sorkin, E., Roth, W., Kocher, V., and Erlenmeyer, H., *Experientia*, **7**, 257–58 (1951)
43. Lutz, A., *Ann. inst. Pasteur*, **76**, 150–67 (1949)
44. Youmans, G. P., Raleigh, G. W., and Youmans, A. S., *J. Bact.*, **54**, 409–16 (1947)
45. Ivanovics, G., *Proc. Soc. Exptl. Biol. Med.*, **70**, 462–63 (1949)
46. Brun, J., Viallier, J., and Kalb, J. C., *Compt. rend. soc. biol.*, **145**, 390–93 (1951)
47. King, T. E., Stearman, R. L., and Cheldelin, V. H., *J. Am. Chem. Soc.*, **70**, 3969 (1948)
48. Wang, C. H., King, T. E., Cheldelin, V. H., and Christensen, B. E., *J. Biol. Chem.*, **188**, 753–58 (1951)
49. Wang, C. H., Cheldelin, V. H., King, T. E., and Christensen, B. E., *J. Biol. Chem.*, **188**, 759–61 (1951)
50. Davis, B., *J. Exptl. Med.*, **94**, 243–54 (1951)
51. Snyder, J. C., and Davis, B. D., *Federation Proc.*, **10**, 419 (1951)
51a. Yasue, Y., *Science (Japan)*, **19**, 423 (1949)
52. Sauberlich, H. E., *Federation Proc.*, **8**, 247–48 (1949)
53. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H., *J. Biol. Chem.*, **185**, 399–409 (1950)
54. Greenspan, E. M., Goldin, A., and Schoenbach, E. B., *Cancer*, **4**, 619–25 (1951)
55. Nichol, C. A., and Welch, A. D., *Proc. Soc. Exptl. Biol. Med.*, **74**, 403–11 (1950)
56. Ellison, R. R., Wolfe, S., Lichtman, H., Ginsberg, V., and Watson, J., *Proc. Soc. Exptl. Biol. Med.*, **76**, 366–70 (1950)
57. Kline, I. T., and Dorfman, R. I., *Proc. Soc. Exptl. Biol. Med.*, **76**, 203–5 (1951)
58. Burchenal, J. H., and Babcock, G. M., *Proc. Soc. Exptl. Biol. Med.*, **76**, 382–84 (1951)
59. Burchenal, J. H., Johnston, S. F., and Waring, G. B., *Proc. Soc. Exptl. Biol. Med.*, **78**, 348–51 (1951)
59a. Weygand, F., Wacker, A., Mann, H. J., and Rowold, E., *Z. Naturforsch.*, **6b**, 174–75 (1951)
60. Broquist, H. P., Fahrenbach, M. J., Brockman, J. A., Jr., Stokstad, E. L. R., and Jukes, T. H., *J. Am. Chem. Soc.*, **73**, 3535–36 (1951)
61. Broquist, H. P., and Jukes, T. H., *Proc. Soc. Exptl. Biol. Med.*, **78**, 563–67 (1951)
62. Shive, W., *Some New Aspects of Anti Anemia Factors* (Presented at 120th Meeting of Am. Chem. Soc., New York, N. Y., Sept., 1951; 7th Southwest Regional Meeting Am. Chem. Soc., Austin, Tex., Dec., 1951)
63. Silverman, M., and Keresztesy, J. C., *J. Am. Chem. Soc.*, **73**, 1897 (1951)
64. Keresztesy, J. C., and Silverman, M., *J. Am. Chem. Soc.*, **73**, 5510 (1951)
65. Minnich, V., Moore, C. V., Smith, D. E., and Gladden, V. E., *Arch. Path.*, **50**, 787–99 (1950)
66. Higgins, G. M., Woods, K. A., *Proc. Staff Meetings Mayo Clinic*, **24**, 238–42 (1949)
67. Dittmar, C., *Z. Krebsforsch.*, **57**, 621–25 (1951)

68. Girdwood, R. H., *Brit. J. Nutrition*, **5**, 1–10 (1951)
69. Skipper, H. E., Chapman, J. B., and Bell, M. J., *Cancer Research*, **11**, 161–63 (1951)
70. Law, L. W., *Proc. Soc. Exptl. Biol. Med.*, **77**, 340–44 (1951)
71. Burchenal, J. H., Karnofsky, D. A., Kingsley-Pillers, E. M., Southam, C. M., Laird Myers, W. P., Escher, G. C., Craver, L. F., Dargeon, H. W., and Rhoads, C. P., *Cancer*, **4**, 549–69 (1951)
72. Williams, J. N., Jr., *Proc. Soc. Exptl. Biol. Med.*, **78**, 206–8 (1951)
73. Dinning, J. S., Keith, C. K., Davis, P. L., and Day, P. L., *Arch. Biochem.*, **27**, 89–93 (1950)
74. Williams, J. N., Jr., *J. Biol. Chem.*, **191**, 123–27 (1951); **192**, 81–85 (1951)
75. Samarth, K. D., Baxi, A. J., Baliga, B. R., and Venkataraman, P. R., *Current Sci. (India)*, **20**, 120–21 (1951)
76. Skipper, H. E., Mitchell, J. H., Jr., and Bennett, L. L., Jr., *Cancer Research*, **10**, 510–12 (1950)
77. Goldthwait, D. A., and Bendich, A., *Federation Proc.*, **10**, 190 (1951)
78. Rosenbaum, R. M., and Velardo, J. T., *Nature*, **168**, 424 (1951)
79. King, C. T. G., and Velardo, J. T., *Federation Proc.*, **10**, 208 (1951)
80. Gaines, D. S., and Totter, J. R., *Proc. Soc. Exptl. Biol. Med.*, **74**, 558–61 (1950)
81. Gaines, D. S., and Broquist, H. P., *Federation Proc.*, **10**, 186 (1951)
82. Gaines, D. S., Broquist, H. P., and Williams, W. L., *Proc. Soc. Exptl. Biol. Med.*, **77**, 247–49 (1951)
83. Bakerman, H. A., Silverman, M., and Daft, F. S., *J. Biol. Chem.*, **188**, 117–23 (1951)
84. Silverman, M., Gardiner, R. C., and Bakerman, H. A., *J. Biol. Chem.*, **194**, 815–21 (1952)
85. Kidder, G. W., Dewey, V. C., and Parks, R. E., Jr., *Proc. Soc. Exptl. Biol. Med.*, **78**, 88–91 (1951)
86. Modest, E. J., Foley, G. E., Pechet, M. M., and Farber, S., *J. Am. Chem. Soc.*, **74**, 855–56 (1952)
87. Cosulich, D. B., Seeger, D. R., Fahrenbach, M. J., Roth, B., Mowat, J. H., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, **73**, 2554–57 (1951)
88. Roth, B., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, **73**, 2864–68 (1951)
89. Roth, B., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, **72**, 1914–18 (1950)
90. Roth, B., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, **73**, 2869–71 (1951)
91. Angier, R. B., Boothe, J. H., Mowat, J. H., Waller, C. W., and Semb, J., *J. Am. Chem. Soc.*, **74**, 408–11 (1952)
92. Elion, G. B., and Hitchings, G. H., *J. Biol. Chem.*, **188**, 611–21 (1951)
93. Elion, G. B., and Hitchings, G. H., and VanderWerff, H., *J. Biol. Chem.*, **192**, 505–18 (1951)
94. Collier, H. O. J., and Waterhouse, P. D., *Ann. Trop. Med. Parasitol.*, **44**, 273–80 (1950)
95. Collier, H. O. J., and Hall, I. F., *Ann. Trop. Med. Parasitol.*, **45**, 51–57, 58–61 (1951)
96. Greenberg, J., and Richeson, E. M., *Proc. Soc. Exptl. Biol. Med.*, **77**, 174–76 (1951)

97. Greenberg, J., Boyd, B. L., and Josephson, E. S., *J. Pharmacol. Exptl. Therap.*, **94,** 60–64 (1948)
98. Greenberg, J., *J. Pharmacol. Exptl. Therap.*, **97,** 484–87 (1949); **99,** 444–49 (1950); *Proc. Soc. Exptl. Biol. Med.*, **71,** 306–8 (1949)
99. Falco, E. A., Hitchings, G. H., Russell, P. B., and VanderWerff, H., *Nature*, **164,** 107–8 (1949)
100. Daniel, L. J., Norris, L. C., Scott, M. L., and Heuser, G. F., *J. Biol. Chem.*, **169,** 689–97 (1947)
101. Woolley, D. W., Singer, E. A., and Smith, N., *J. Exptl. Med.*, **93,** 13–24 (1951)
102. Woolley, D. W., *Proc. Soc. Exptl. Biol. Med.*, **75,** 745–46 (1950)
103. Woolley, D. W., and Pringle, A., *J. Biol. Chem.*, **194,** 729–46 (1952)
104. Hendlin, D., and Soars, M. H., *J. Bact.*, **62,** 633–38 (1951); *Federation Proc.*, **10** 384 (1951)
105. Beiler, J. M., Moss, J. N., and Martin, G. J., *Science*, **114,** 122–23 (1951)
105a. Emerson, G., Holly, F. W., Shunk, C. H., Brink, N. G., and Folkers, K., *J. Am. Chem. Soc.*, **73,** 1069–70 (1951)
106. Emerson, G., Brink, N. G., Holly, F. W., Konivsky, F., Heyl, D., and Folkers, K., *J. Am. Chem. Soc.*, **72,** 3084–85 (1950)
107. Cooperman, J. M., and Tabenkin, B., *Federation Proc.*, **10,** 175 (1951)
108. Dion, H. W., Colkins, D. G., and Pfiffner, J. J., *J. Am. Chem. Soc.*, **74,** 1108 (1952)
109. Wright, L. D., Cressen, E. L., Skeggs, H. R., Peck, R. L., Wolf, D. E., Wood, T. R., Valiant, J., and Folkers, K., *Science*, **114,** 635–36 (1951)
110. Wright, L. D., Skeggs, H. R., and Cresson, E. L., *J. Am. Chem. Soc.*, **73,** 4144–45 (1951)
111. Whiteside-Carlson, V., Starnes, W. R., Rosano, C. L., and Carlson, W. W., *Proc. Soc. Exptl. Biol. Med.*, **77,** 344–8 (1951)
112. Pope, H., *J. Bact.*, **63,** 39–45 (1952)
113. Drekter, L., Scheiner, J., Ritter, E. de, and Rubin, S. H., *Federation Proc.*, **10,** 381 (1951)
114. Launer, H. F., and Fraenkel-Conrat, H., *J. Biol. Chem.*, **193,** 125–32 (1951)
115. Feigelson, P., Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, **189,** 361–69 (1951)
116. Gaebler, O. H., and Beher, W. T., *J. Biol. Chem.*, **188,** 343–49 (1951)
117. Cooperman, J. M., Rubin, S. H., and Tabenkin, B., *Proc. Soc. Exptl. Biol. Med.*, **76,** 18–20 (1951)
118. Berger, J., Jampolsky, L. M., and Goldberg, M. W., *Arch. Biochem.*, **22,** 476–78 (1949)
119. Schlenk, F., Hellstrom, H., and Euler, H. von, *Ber.*, **71,** 1471–79 (1938)
120. Pullman, M. E., *Federation Proc.*, **10,** 234–35 (1951)
121. McRorie, R. A., Masley, P. M., and Williams, W. L., *Arch. Biochem.*, **27,** 471–73 (1950)
122. Brown, G. M., Craig, J. A., and Snell, E. E., *Arch. Biochem.*, **27,** 473–75 (1950)
123. McRorie, R. A., and Williams, W. L., *J. Bact.*, **61,** 737–45 (1951)
124. Cheldelin, V. H., and King, T. E., *Bound Forms of Pantothenic Acid* (7th Southwest Regional Meeting, Am. Chem. Soc., Austin, Tex., Dec., 1951)
125. Snell, E. E., Brown, G. M., Peters, V. J., Craig, J. A., Wittle, E. L., Moore, J. A., McGlohon, V. M., and Bird, O. D., *J. Am. Chem. Soc.*, **72,** 5349–50 (1950)
126. King, T. E., and Strong, F. M., *J. Biol. Chem.*, **189,** 325–33 (1951)

127. Drell, W., and Dunn, M. S., *Arch. Biochem. Biophys.*, **33,** 110–19 (1951)
128. Lansford, E. M., Jr., and Shive, W., *J. Biol. Chem.*, **194,** 329 (1952)
129. Baddiley, J., and Thain, E. M., *J. Chem. Soc.*, 246–51 (1951)
130. King, T. E., and Strong, F. M., *Science*, **112,** 562–65 (1950)
131. Lichstein, H. C., and Gilfillan, R. F., *Proc. Soc. Exptl. Biol. Med.*, **77,** 459–61 (1951)
132. Jones, R. G., and Kornfeld, E. C., *J. Am. Chem. Soc.*, **73,** 107–9 (1951)
133. Glazer, H. S., Fichter, B., Mueller, J. F., and Vilter, R. W., *Arch. Biochem. Biophys.*, **32,** 424–27 (1951)
134. Glazer, H. S., Mueller, J. F., Thompson, C., Hawkins, V. R., and Vilter, R. W., *Arch. Biochem. Biophys.*, **33,** 243–51 (1951)
135. Shanmuga Sundaram, E. R. B., Ranganathan, G., and Sarma, P. S., *Current Sci. (India)*, **20,** 122–24 (1951)
136. Heyl, D., Luz, E., Harris, S. A., and Folkers, K., *J. Am. Chem. Soc.*, **74,** 414–16 (1952)
137. Kearney, E. B., *J. Biol. Chem.*, **194,** 747–54 (1952)
138. Morris, H. P., and Robertson, W. van B., *J. Natl. Cancer Inst.*, **3,** 479–89 (1943)
139. Stoerk, H. C., and Emerson, G. A., *Proc. Soc. Exptl. Biol. Med.*, **139,** 703–4 (1949)
140. Holly, F. W., Peel, E. W., Mozingo, R., and Folkers, K., *J. Am. Chem. Soc.*, **72,** 5416–17 (1950)
141. Aposhian, H. V., and Lambooy, J. P., *Proc. Soc. Exptl. Biol. Med.*, **78,** 197–99 (1951)
142. Lambooy, J. P., *J. Biol. Chem.*, **188,** 459–62 (1951)
143. Lambooy, J. P., *Federation Proc.*, **10,** 386 (1951)
144. Emerson, G. A., and Folkers, K., *J. Am. Chem. Soc.*, **73,** 2398–99, 5383–86 (1951)
145. Palleres, E. S., and Garza, H. M., *Arch. Biochem.*, **22,** 63 (1949)
146. Emerson, G. A., and Casey, D., *Federation Proc.*, **10,** 381 (1951)
147. Eusebi, A. J., and Cerecedo, L. R., *Science*, **110,** 162 (1949)
148. Wilson, A. W., and Harris, S. A., *J. Am. Chem. Soc.*, **71,** 2231–33 (1949)
149. Rabinowitz, J. C., and Snell, E. E., *Arch. Biochem. Biophys.*, **33,** 472–81 (1951)
150. Woolley, D. W., *J. Biol. Chem.*, **191,** 43–54 (1951)
151. Ulrich, J. A., and Fitzpatrick, T. B., *Proc. Soc. Exptl. Biol. Med.*, **76,** 346–49 (1951)
152. Velluz, L., and Herbain, M., *J. Biol. Chem.*, **190,** 241–44 (1951)
153. Schopfer, W. H., Bein, M. L., Beeson, G., and Eichin, R., *Z. Vitaminforsch.*, **23,** 47–58 (1951)
154. Ryman, B. E., and Walsh, E. O., *Nature*, **167,** 770 (1951)
155. Mitchell, H. K., and Houlahan, M. B., *Federation Proc.*, **6,** 506–9 (1947)
156. Mitchell, H. K., Houlahan, M. B., and Nyc, J. F., *J. Biol. Chem.*, **172,** 525–29 (1948)
157. Wright, L. D., Valentik, K. A., Spicer, D. S., Huff, J. W., and Skeggs, H. R., *Proc. Soc. Exptl. Biol. Med.*, **75,** 293–97 (1950)
158. Strandskov, F. B., and Wyss, O., *J. Bact.*, **50,** 237 (1945)
159. Hitchings, G. H., Elion, G. B., and Falco, E. A., *J. Biol. Chem.*, **185,** 643–49 (1950)
160. Hitchings, G. H., Elion, G. B., Falco, E. A., Russell, P. B., Sherwood, M. B., and VanderWerff, H., *J. Biol. Chem.*, **183,** 1–9 (1950)
161. Hitchings, G. A., Elion, G. B., Falco, E. A., Russell, P. B., and VanderWerff, H., *Ann. N. Y. Acad. Sci.*, **52,** 1318–35 (1950)

162. Weygand, F., Wacker, A., and Grisebach, H., *Z. Naturforsch.*, **6b**, 177–78 (1951)
163. Hitchings, G. H., Elion, G. B., and VanderWerff, H., *J. Biol. Chem.*, **174**, 1037–38 (1948)
164. Roberts, M., and Visser, D. W., *J. Biol. Chem.*, **194**, 695–701 (1952)
165. Fukuhara, T. K., and Visser, D. W., *J. Biol. Chem.*, **190**, 95–101 (1951)
166. Commoner, B., and Mercer, F. L., *Arch. Biochem. Biophys.*, **35**, 278–89 (1952); *Nature*, **168**, 113–14 (1951)
167. Wolff, R., and Karlin, R., *Compt. rend. soc. biol.*, **145**, 318–20 (1951)
168. Rafelson, M. E., Jr., Pearson, H. E., and Winzler, R. J., *Proc. Soc. Exptl. Biol. Med.*, **76**, 689–92 (1951)
169. DiCarlo, F. J., Schultz, A. S., and Kent, A. M., *J. Biol. Chem.*, **194**, 769–844 (1952)
170. Spizizen, J., *J. Infectious Diseases*, **73**, 212–21 (1943)
171. Elion, G. B., and Hitchings, G. H., *J. Biol. Chem.*, **187**, 511–22 (1950)
172. Skipper, H. E., Mitchell, J. H., Jr., Bennett, L. L., Jr., Newton, M. A., Simpson, L., and Edison, M., *Cancer Research*, **11**, 145–49 (1951)
173. Biesele, J. J., Berger, R. E., Wilson, A. Y., Hitchings, G. H., and Elion, G. B., *Cancer*, **4**, 186–97 (1951)
174. Kidder, G. W., Dewey, V. C., Parks, R. E., Jr., and Woodside, G. L., *Cancer Research*, **11**, 204–11 (1951)
175. Finkelstein, M., and Thomas, P. A., *Cancer Research*, **11**, 801–6 (1951)
176. Goldin, A., Greenspan, E. M., and Schoenbach, E. B., *J. Natl. Cancer Inst.*, **11**, 319–38 (1950)
177. Gellhorn, A., Kream, J., and Hirschberg, E., *Federation Proc.*, **10**, 297–98 (1951)
178. Roush, H., and Norris, E. R., *Arch. Biochem.*, **29**, 124–29 (1950)
179. Kidder, G. W., Dewey, V. C., and Parks, R. E., Jr., *Federation Proc.*, **10**, 207 (1951)
180. Drell, W., *Federation Proc.*, **10**, 177–78 (1951)
181. Friend, C., *Federation Proc.*, **10**, 354 (1951)
182. Moore, A. E., and Friend, C., *Federation Proc.*, **10**, 365–66 (1951)
183. Elion, G. B., Burgi, E., and Hitchings, G. H., *J. Am. Chem. Soc.*, **74**, 411–14 (1952)
184. Woolley, D. W., and Shaw, E., *J. Biol. Chem.*, **189**, 401–10 (1951)
185. Shaw, E., and Woolley, D. W., *J. Biol. Chem.*, **194**, 641–654 (1952)
186. Vigneaud, V. du, McKennis, H., Jr., Simmonds, S., Dittmer, K., and Brown, G. B., *J. Biol. Chem.*, **159**, 385–94 (1945); Ferger, M. F., and duVigneaud, V., *J. Biol. Chem.*, **179**, 61–65 (1949)
187. Dittmer, K., Ellis, G., McKennis, H., Jr., and duVigneaud, V., *J. Biol. Chem.*, **164**, 761–71 (1946)
188. Beerstecher, E., Jr., and Shive, W., *J. Biol. Chem.*, **164**, 53–61 (1946)
189. Drea, W. F., *J. Bact.*, **56**, 257–58 (1948)
190. Pope, H., *J. Bact.*, **58**, 223–28 (1949)
191. Beerstecher, E., Jr., and Shive, W., *J. Biol. Chem.*, **167**, 49–52 (1947)
192. Dittmer, K., *J. Am. Chem. Soc.*, **71**, 1205–7 (1949)
193. Garst, R. G., Campaigne, E., and Day, H. G., *J. Biol. Chem.*, **180**, 1013–20 (1949)
194. Clark, D. A., and Dittmer, K., *J. Biol. Chem.*, **173**, 313–21 (1948)
195. Dittmer, K., *Ann. N. Y. Acad. Sci.*, **52**, 1277 (1950)
196. Herz, W., Dittmer, K., and Cristol, S. J., *J. Am. Chem. Soc.*, **70**, 504–7 (1948)

197. Mitchell, H. K., and Niemann, C., *J. Am. Chem. Soc.*, **69,** 1232 (1947)
198. Elliot, D. F., Fuller, A. T., and Harington, C. R., *J. Chem. Soc.*, 85–89 (1948)
199. Boyer, P. D., Evans, R. J., and Phillips, P. H., *J. Pharmacol. Exptl. Therap.*, **73,** 177–85 (1941)
200. Mitchell, H. K., and Niemann, C., *J. Am. Chem. Soc.*, **69,** 1232 (1947)
201. Martin, G. J., and Moss, J. N., *Am. J. Pharm.*, **121,** 169–72 (1949)
202. Atkinson, D. E., Melvin, S., and Fox, S. W., *Arch. Biochem. Biophys.*, **31,** 205–11 (1951)
203. Spiegelman, S., and Halvorson, H. O., in Armstrong, M. D., and Lewis, J. D., *J. Biol. Chem.*, **190,** 461–66 (1951)
204. Armstrong, M. D., and Lewis, J. D., *J. Biol. Chem.*, **190,** 461–66 (1951)
205. Armstrong, M. D., and Lewis, J. D., *J. Biol. Chem.*, **188,** 91–95 (1951)
206. Kleinzeller, A., and Kubie, G., *Chem. Listy*, **44,** 210–11 (1950)
207. Marnay, C., *Bull. soc. chim. biol.*, **33,** 174–178 (1951); *Chem. Abstracts*, **44,** 3074a (1951)
208. Volcani, B. E., Sicher, S., and Lichtenstein, N., *J. Biol. Chem.*, **192,** 543–49 (1951)
209. Beerstecher, E., Jr., and Shive, W., *J. Biol. Chem.*, **167,** 527–34 (1947)
210. Burckhalter, J. H., and Stephens, V. C., *J. Am. Chem. Soc.*, **73,** 56–58 (1951)
211. Harper, H. A., Furst, A., and Morris, F. D., *Wasmann J. Biol.*, **8,** 299–302 (1950)
212. Frieden, E., Stansel, H. C., Jr., and Dittmer, K., *Federation Proc.*, **10,** 184 (1951)
213. Elliot, D. F., and Harrington, C. R., *J. Chem. Soc.*, 85–89 (1948)
214. Furst, A., Harper, H. A., and Siewald, R. J., Morris, M. D., and Neve, R. A., *Arch. Biochem.*, **31,** 190–96 (1951)
215. Woolley, D. W., Smith, N., and Singer, E. A., *J. Biol. Chem.*, **185,** 293–305 (1950)
216. Molho, D., and Molho-Lacroix, L., *Bull. soc. chim. biol.*, **32,** 680–86, 687–95, 969–76 (1950)
217. Molho, D., and Molho-Lacroix, L., *Compt. rend.*, **233,** 1067–69 (1951)
218. Moersch, G. W., Rebstock, M. C., Moore, A. C., and Hylander, D. P., *J. Am. Chem. Soc.*, **74,** 565–66 (1952)
219. Hubbard, D. E., and Schmidt, E. G., *Proc. Soc. Exptl. Biol. Med.*, **77,** 766–68 (1951)
220. Jones, J. H., Foster, C., Henle, W., and Alexander, D., *Arch. Biochem.*, **11,** 481–87 (1946)
221. Rasmussen, A. F., Jr., Clark, P. F., Smith, S. C., and Elvehjem, C. A., *Bact. Proc.*, 93 (1951)
222. Tatsuoka, S., and Hitomi, H., *J. Pharm. Soc. Japan*, **71,** 871–74 (1951); *Chem. Abstracts*, **46,** 4002–3 (1951)
223. Beerstecher, E., Jr., and Edmonds, E. J., *J. Biol. Chem.*, **192,** 497–504 (1951); Beerstecher, E., Jr., *Some Aspects of the Intermediary Metabolism of Tryptophan and Pantothenic Acid in Bacteria* (Doctoral thesis, Univ. of Texas, Austin, Tex., 1948)
224. Hodgson, R., Peterson, W. H., and Ricker, A. J., *J. Bact.*, **62,** 445–58 (1951)
225. Dunn, F. W., and Dittmer, K., *J. Biol. Chem.*, **188,** 263–72 (1951)
226. Dyer, H. M., *J. Biol. Chem.*, **124,** 519–24 (1938)
227. Harris, J. S., and Kohn, H. I., *J. Pharmacol.*, **73,** 383–400 (1941)
228. Roblin, R. O., Jr., Lampen, J. O., English, J. P., Cole, Q. P., and Vaughan, J. R., *J. Am. Chem. Soc.*, **67,** 290–94 (1945)

229. Goering, H. L., Cristol, S. J., and Dittmer, K., *J. Am. Chem. Soc.*, **70,** 3314–16 (1948)
230. Harding, W. M., and Shive, W., *J. Biol. Chem.*, **174,** 743–55 (1948)
231. Daniel, E. E., *J. Cellular Comp. Physiol.*, **36,** 41–57 (1950)
232. Miller, H. K., and Waelsch, H., *Nature*, **169,** 30–31 (1952)
233. Misani, F., and Reiner, L., *Arch. Biochem.*, **27,** 234–35 (1950)
234. Bentley, H. R., McDermott, E. F., Moran, T., Pace, J., and Whitehead, J. K., *Proc. Roy. Soc.* (*London*), [B]**137,** 402–17 (1950)
235. Reiner, L., Misani, F., and Weiss, P., *Arch. Biochem.*, **25,** 447–49 (1950)
236. Gershoff, S. N., and Elvenhjem, C. A., *J. Nutrition*, **45,** 451–58 (1951)
237. Gershoff, S. N., and Elvenhjem, C. A., *J. Biol. Chem.*, **192,** 569–74 (1951)
238. Heathcote, J. G., and Pace, J., *Nature*, **166,** 353–55 (1950)
239. Stekol, J. A., and Weiss, K. W., *J. Biol. Chem.*, **179,** 1049–56 (1944)
240. Stekol, J. A., and Weiss, K. W., *Proc. Soc. Exptl. Biol. Med.*, **77,** 213–14 (1951)
241. Farber, E., Simpson, M. V., and Tarver, H., *J. Biol. Chem.*, **182,** 91–99 (cf. 81–89) (1950)
242. Jensen, D., Chaikoff, I. L., and Tarver, H., *J. Biol. Chem.*, **192,** 395–403 (1951)
243. Stekol, J. A., and Weiss, K. W., *J. Biol. Chem.*, **185,** 577–83 (1950)
244. Levine, M., and Tarver, H., *J. Biol. Chem.*, **192,** 835 (1951); Levine, M., *Federation Proc.*, **10,** 214 (1951)
245. Swendseid, M. E., Swanson, A. L., and Bethall, F. H., *Federation Proc.*, **10,** 257 (1951)
246. Shaffer, C. B., and Critchfield, F. H., *J. Biol. Chem.*, **174,** 489–93 (1948)
247. Travers, J. J., and Cerecedo, L. R., *Proc. Soc. Exptl. Biol. Med.*, **76,** 497–99 (1951)
248. Ackermann, W. W., *Federation Proc.*, **10,** 400 (1951); *J. Exptl. Med.*, **93,** 337 (1951)
249. Brown, G. C., and Ackermann, W. W., *Federation Proc.*, **10,** 403 (1951)
250. Brown, G. C., Ackermann, W. W., and Schieble, J. H., *Proc. Soc. Exptl. Biol. Med.*, **77,** 367–69 (1951)
251. Ainslie, D. S., *J. Exptl. Med.*, **95,** 9–18 (1951)
252. Moldave, K., Winzler, R. J., and Pearson, H. E., *Federation Proc.*, **10,** 225 (1951)
253. Waelsch, H., Owades, P., Miller, H. K., and Borek, E., *J. Biol. Chem.*, **166,** 272–81 (1946)
254. Borek, E., and Waelsch, H., *Arch. Biochem.*, **14,** 143–47 (1947)
255. McLaren, A. D., and Knight, C. A., *J. Am. Chem. Soc.*, **73,** 4478–79 (1951)
256. Good, N., Heilbronner, R., and Mitchell, H. K., *Arch. Biochem.*, **28,** 464–65 (1950)
257. Volcani, B. E., and Snell, E. E., *J. Biol. Chem.*, **174,** 893–902 (1948)
258. Horowitz, N. H., and Srb, A. M., *J. Biol. Chem.*, **174,** 371–78 (1948)
259. Teas, H. J., *J. Biol. Chem.*, **190,** 369–75 (1951)
260. Miller, E. J., and Harrison, J. S., *Nature*, **166,** 1035 (1950)
261. Simmonds, S., Harris, J. I., and Fruton, J. S., *J. Biol. Chem.*, **188,** 251–62 (1951)
262. Frieden, E., Hsu, L. T., and Dittmer, K., *J. Biol. Chem.*, **192,** 425–33 (1951)
263. Ackerman, W. W., and Kleinschmidt, E., *J. Biol. Chem.*, **192,** 421–28 (1951)

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